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THE EFFECT OF FORCING FLUIDS UPON SURVIVAL AFTER BILATERAL EPINEPHRECTOMY

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From the Osborn Zoölogical Laboratory, Yale University

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Previous work by several investigators has shown that the administration of certain solutions may result in prolongation of life beyond the usual period in epinephrectomized animals. Thus, Stewart and Rogoff (1925), working on dogs, report that intravenous injections of Ringer's solution and dextrose prolong life. Zwemer (1925), working on cats in this laboratory, was able to keep his double-operated animals alive for considerable periods by administering a 5 per cent solution of glucose orally. This work suggested the possibility that forcing fluids containing various substances, or even water alone, might prove beneficial in the treatment of adrenal insufficiency following gland removal. Both Zwemer (1926) and Swingle (1926), in this laboratory, have noted and commented upon the symptoms of dehydration which appear after bilateral epinephrectomy in cats, and the fact that animals presenting marked signs of adrenal insufficiency eagerly drink large quantities of water up to within a few hours of death. Their observations lent further support to the idea that forcing fluids might prove efficacious.

The writer takes this opportunity of expressing his obligation to Prof. W. W. Swingle for suggesting the problem and for kindly criticism during the progress of the work.

The oral method for administration of fluids was adopted. By means of the stomach tube, solutions were given to the animal beginning immediately after the second operation, and the treatment was continued until the appearance of symptoms immediately premonitory to death. The passage of the tube when an animal was in, or verging on, coma, was found to be useless, as the excitation almost invariably led to convulsions and death within a few minutes. In order to pass the stomach tube—a rubber catheter of suitable diameter—the animals were placed in a wooden box so constructed that the head alone protruded. The box was of such size as to allow the animals a minimum of movement coincident with comfort. A rubber gag, composed of a short piece of tubing threaded on a stout cord, was next inserted into the mouth of the animal and the ends of the cord tied behind the ears. This prevented puncturing of the tube due to

TABLE 1

NUMBER	SEX	DAYS ELAPSED BETWEEN FIRST AND SECOND OPERATION	SURVIVAL AFTER SECOND OPERATION	COMPLICATIONS
				Section I
Ringer's solution. Treatment: 50 cc. Ringer's solution twice daily, 50 cc. milk once daily.				
			hours	
1	Male	9	216.0	None
2	Female	8	132.0	None
3	Female	7	62.0	Abnormal, large, cyst at site of kidney, complicating operation
				Section II
Ringer's solution (each constituent double strength). Treatment: 50 cc. Ringer's solution twice daily, 50 cc. milk once a day.				
4	Male	7	188.0	None
5	Female	7	96.0	None
				Section III
Solution "1": Magnesium lactate, 0.9 per cent; strontium chloride, 0.024 per cent; sodium (acid) phosphate, 0.042 per cent. Treatment: 50 cc. solution "1" twice daily, 50 cc. milk once daily.				
6	Female	9	341.0	Stitch infection
7	Male	7	107.0	None
8	Male	7	196.0	None
9	Female	7	125.0	None
10	Female	3	91.5	Wound infection
				Section IV
Solution "1-A": Magnesium lactate, 0.9 per cent; strontium lactate, 0.024 per cent; sodium (acid) phosphate, 0.042 per cent. Treatment: 50 cc. solution "1-A" three times daily, 50 cc. milk once daily.				
11	Female	7	162.5	None
12	Male	7	137.5	Wound infection
				Section V
Solution "1-B": Magnesium sulphate, 0.9 per cent; strontium chloride, 0.024 per cent; sodium sulphate, 0.042 per cent. Treatment: 50 cc. solution "1-B" twice daily, 50 cc. milk once daily.				
13	Female	7	18.0	Broke ligature-hemorrhage
14	Male	5	68.0	None
15	Male	5	69.5	None

TABLE 1—*Continued*

AVERAGE PROLONGATION	WEIGHT AFTER SECOND OPERATION	WEIGHT AT DEATH	TOTAL LOSS
	Section I		
	grams	*grams*	*grams*
174 hours excluding no. 3	Not recorded	Not recorded	Not recorded
	Not recorded	Not recorded	Not recorded
	Not recorded	Not recorded	Not recorded
	Section II		
	grams	*grams*	*grams*
142 hours	4350	4020	330
	Not recorded	Not recorded	Not recorded
	Section III		
192.25 hours, excluding no. 10	Not recorded	Not recorded	Not recorded
	Not recorded	Not recorded	Not recorded
	3225	3025	200
	1900	1850	50
	2250	2200	50
	Section IV		
150 hours	2260	2220	40
	2428	2190	238
	Section V		
68.75 hours, excluding no. 13	Not recorded	Not recorded	Not recorded
	2953	2645	308
	2983	2690	293

TABLE 1—*Continued*

NUMBER	SEX	DAYS ELAPSED BETWEEN FIRST AND SECOND OPERATION	SURVIVAL AFTER SECOND OPERATION	COMPLICATIONS
				Section VI
				Solution "1-C": Magnesium sulphate, 0.9 per cent; strontium chloride, 0.024 per cent; sodium (acid) phosphate, 0.042 per cent; ammonium chloride, 0.05 per cent; sodium chloride, 0.042 per cent; sodium bicarbonate, 0.042 per cent; glucose, 0.9 per cent.
				Treatment: 30 cc. solution "1-C" twice daily, 50 cc. milk once daily.
			hours	
16	Male	5	177.0	None
17	Male	8	144.0	None
18	Female	9	47.0	Accidentally killed
				Section VII
				Sodium chloride, 0.9 per cent.
				Treatment: 30 cc. sodium chloride three times daily, 50 cc. milk once daily.
19	Male	13	168.0	None
20	Male	4	91.5	None
21	Female	7	187.5	None
				Section VIII
				Sodium bicarbonate, 0.5 per cent.
				Treatment: 30 cc. sodium bicarbonate twice daily, 50 cc. milk once daily.
22	Male	8	178.5	None
23	Female	8	163.0	None
24	Female	4	168.0	None
25	Male	7	80.0	Intestinal disorders—extreme diarrhea
				Section IX
				Sodium acetate, 3.5 per cent.
				Treatment: 50 cc. sodium acetate three times daily, 50 cc. milk once daily.
26	Female	7	42.0	Vomited everything given
27	Male	12	65.5	Stitch infection, vomited

chewing or biting. The stomach tube could then be passed without difficulty. Following a few passages of the tube, the animals took it quite readily with very little struggling. Without such an apparatus it was found to be practically impossible to administer solutions to a cat by stomach tube, and this arrangement is of particular value as the animals struggle very little—struggling and exertion always tending to aggravate the symptoms due to the extirpation of the glands.

Upon death the animals were autopsied for the presence of accessory

TABLE 1—*Concluded*

AVERAGE PROLONGATION	WEIGHT AFTER SECOND OPERATION	WEIGHT AT DEATH	TOTAL LOSS
		Section VI	
106.5 hours, excluding no. 18	2335	2310	25
	3565	3310	255
	2215	2202	13
		Section VII	
152.2 hours	3584	3192	392
	2770	2650	120
	2240	1950	290
		Section VIII	
147.3 hours	2870	2750	120
	1835	1740	115
	3270	3160	110
	2785	2655	130
		Section IX	
53.7 hours	2760	2600	160
	Not recorded	Not recorded	Not recorded

glands or any portion of glandular tissue not removed upon operation. Data upon such an animal showing either an accessory cortical gland, or bit of gland left behind at operation, were discarded as valueless in this study. In calculating weight losses, the weights were taken immediately after the second operation and at death, the difference in weight being recorded in the tables as the total weight loss.

Five to ten days was allowed to elapse between first and second operations. Zwemer (1926) and Swingle (1926) both observed that male cats

which have had both adrenals removed in two stages, 6 to 10 days apart, seldom survive over 60 hours after the second operation. Their data were obtained from animals operated upon during the fall and winter months. The present experiments were done simultaneously with those recorded in their papers and their data for untreated animals serve as checks for the treated animals reported upon here.

Previous investigation by Elliott and Tuckett (1906), Marshal and Davis (1916), and Zwemer (1925) had shown the necessity for adopting a definite time interval between the first and second operation when working upon the prolongation of life in bilaterally epinephrectomized animals. The investigators mentioned showed that if a considerable interval of time is allowed to elapse between the first and second operations the life-span of cats is considerably prolonged. Just why long intervals between operations should enable an animal to survive a greater length of time is unknown. It seems probable that minute bits of accessory cortical tissue may thus have time to hypertrophy and function to some degree, although at present there are no available data to substantiate this conclusion. It has long been known that when both adrenals are removed at one sitting death quickly results.

Various solutions were employed. The nature of the solution and data regarding each experiment are recorded in the tables. The food of the double operated animals was milk and this was given by stomach tube. Cats suffering from adrenal insufficiency rarely eat voluntarily after the first thirty hours following operation, and seem to have an especial loathing for meat.

None of the female cats employed in these experiments were pregnant. This is a point which must always be taken into consideration when dealing with any problem concerning survival periods of bilaterally epinephrectomized animals. H. A. Stewart (1913) was the first to clearly demonstrate that pregnancy definitely prolongs the life of the operated cats. More recently Stewart and Rogoff (1925) have confirmed this result in dogs.

In section I of the table, data are presented showing that the oral administration of Ringer's solution prolongs life considerably. The average life span of double operated animals—with an interval of seven days between operations, is about 60 hours. This figure is based on the data obtained from the records of one hundred animals operated upon in this laboratory by Swingle (1926) and Zwemer (1925–26). The animals were operated upon during the fall and winter months. If we exclude animal 3, obviously an abnormal case owing to the presence of an enormous cyst on the kidney site, the survival period averaged 174 hours. There can be little question but that Ringer's solution aids in prolonging life in epinephrectomized cats.

It is interesting to note that doubling the percentage of the constituents of Ringer's solution (section II) did not materially increase the life-span of double operated animals.

Study of the table and the various solutions employed, reveals the interesting fact that almost any solution when administered in large quantities is effective in prolonging life. For example, in section VII we find an average prolongation of 152.2 hours for the animals treated with sodium chloride, and in section VIII the data show that sodium bicarbonate is only slightly less effective, giving an average survival of 147.3 hours.

Certain solutions proved more efficacious than others in preserving life. Thus, solution 1, section III of the table, proved very effective, whereas solution 1-B, section V, and sodium acetate, section IX, were ineffective. In the case of sodium acetate, however, the animals had great difficulty holding the material as it had an irritating effect upon the stomach.

Zwemer (1926) observed that 5 per cent solutions of glucose given orally prolonged the life of double operated animals an average of nine days, whereas the untreated controls succumbed within 60 hours. Glucose appears to be more effective than any of the substances employed in the present experiment.

It is not at all clear why forcing fluids should prolong the life of bilaterally epinephrectomized cats. It is evident from the work of Swingle and Zwemer that dehydration is one of the train of symptoms following adrenal removal in cats, and flooding the organism with fluids tends to temporarily relieve this symptom of adrenal insufficiency. The explanation which most readily comes to mind in this connection is that the forcing of fluids and the consequent diuresis flushes some toxic substance out of the bloodstream of the organism. However this may be, it seems evident that the forcing of fluid definitely prolongs the life of bilaterally epinephrectomized cats, but this action is merely palliative and not curative, since all animals so treated eventually succumb with typical symptoms of adrenal insufficiency.

We believe that the period of survival of our treated cats is not due to the time interval between the first and second operation, since this factor was carefully controlled. Animal 16, section VI of the table, in which 5 days elapsed between operations shows a survival period of 177 hours; while no. 17, subjected to the same treatment, and having an 8-day interval between the first and second operations, shows a survival of 144 hours. The same is seen in a comparison of animals 19 and 21, section VII as well as nos. 23 and 24, section VIII. Cases of the converse do occur in the tables, however, as in cat 20, which shows a survival of 91.5 hours with a 4-day interval between operations, as against no. 21. on the same treatment, with a survival of 187.5 hours and an interval of 7 days between the extirpation of the glands. With a reasonable interval between the operations, 5 to 10

days, there does not appear to be any relation whatever between this interval and the length of the survival period. There can be little doubt however, in view of the results of other workers, that the lapse of long intervals between operations tends to prolong the survival period.

As regards the total weight lost after the second operation, it appears to be more closely related to the weight of the animal than to any other factor. At any rate, the animals show no more marked losses in weight under one type of treatment than another. As a rule, the heavier animals with large deposits of fat showed the most marked losses, as would be expected.

SUMMARY

1. The oral administration of large quantities of fluid to bilaterally epinephrectomized cats prolongs life.

2. Solutions of certain substances (i.e., glucose, NaCl, etc.) are more effective than others in maintaining the animal in a normal condition.

3. The explanation why forcing fluids prolongs the life of double operated cats is not entirely clear. It seems to partially relieve the dehydration which is one of the train of symptoms of adrenal insufficiency in cats, and possibly by the consequent diuresis flushes toxic substances out of the organism.

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AN EXPERIMENTAL STUDY OF THE ADRENAL CORTEX

I. The Survival Value of the Adrenal Cortex

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Seventy-seven years ago, Addison (1849) in speaking before the South London Medical Society, stated that he had at least an inkling as to the function of "these hitherto mysterious bodies—the suprarenal capsules." He described a peculiar type of anemia, which was associated with general weakness and an indisposition toward physical and mental exertion, and which terminated with death from exhaustion in a few weeks or months. In the cases that were brought to necropsy there was found a diseased condition of the suprarenal capsules.

In spite of the more than three quarters of a century that have elapsed since he first described this syndrome, we are still without a clear understanding of the rôle of the suprarenal glands in Addison's disease, or as to their function in the normal organism. Recent experimental work indicates that the cortex of the suprarenal is necessary for life and that the medulla of the gland may be removed without serious consequences resulting.

Stewart and Rogoff (1917), (1919) removed one adrenal and sectioned all the nerves joining to the other and found that after five weeks no adrenalin could be demonstrated in blood taken from the adrenal vein. They performed this experiment on dogs, cats, rabbits and monkeys, and their test was sensitive up to 1:1000 millions. It would seem quite evident from their data that adrenalin is not necessary for life, as their animals developed no abnormal symptoms.

Wheeler and Vincent (1917) in experiments on 30 dogs, 22 cats and 4 rabbits, removed one and one-half adrenals and cauterized the medulla from the remaining half. Three weeks to a month later the cauterized gland was removed post mortem and examined for medullary tissue. The examination showed that in "several" cases no medullary tissue was present. While not conclusive, this is further evidence for the importance of the cortex. Houssay and Lewis (1923) curetted out the medulla from one adrenal and removed the other a week or ten days later. Of the twenty-five dogs used a large majority survived until used for some other experiment. Only two animals were brought to a third operation, at which the

curetted gland was removed. The dogs lived for 24 to 26 hours after the removal of this cortical remnant, which had kept them alive for 217 and 232 days respectively.

Wislocki and Crowe (1923), using two dogs, removed all of one adrenal and two-thirds of the other, cauterizing the medulla in the part left in the animal. In addition they removed the abdominal chromaffin body. This procedure left the animals a negligible amount of chromaffin tissue and one-sixth of the original amount of cortex. The animals lived, showing no symptoms until the bit of cortex was removed.

Elman and Rothman (1924) secured results the reverse of this, by ligating the lumbo-adrenal veins and blunt dissection of the small blood vessels of the adrenals. They found that one gland hypertrophied and the other atrophied, the atrophied gland being mostly medullary tissue. The removal of the hypertrophied gland caused death, in spite of the presence of normally staining medulla in the other gland.

We have adopted the technique used by Houssay and Lewis (1923) and have secured essentially the same results with cats, as we showed in a preliminary report (Zwemer, 1924). In our series a larger number of animals were subjected to the final operation at which the surgically produced cortical gland was removed. Death followed its removal, showing that it was responsible for the maintenance of life.

The writer takes this opportunity to express his gratitude to Dr. W. W. Swingle, who so willingly gave encouragement and advice in the carrying out of these studies, and to the Biological Laboratory, Cold Spring Harbor, L. I., N. Y., for facilities during the summer of 1924.

Experimental. Cats were used in all our experiments because infrequency of accessory glands in these animals would tend to make the results more uniform, and it is well known that adrenal extirpation in cats leads to a fatal issue. In more than 100 animals used, only one was found with an accessory gland of cortical material. Moreover, cats have been used extensively in this laboratory for parathyroid work, and their care and feeding have been carefully studied. For animals that remained in the laboratory a long time, (i.e., series C) the standard diet of meat (cooked and raw) and milk was supplemented by fish.

The duration of life after the complete removal of the adrenals depends on the species used, and to a large extent on the time interval between the first and second operations (Elliott, 1914). For the last named reason, the interoperative period should always be given. Fairly uniform results can be secured with a wait of one to two weeks between operations. In all our tables this time interval is listed in the column immediately following the series number and sex of the cat.

The lumbar pathway was used for epinephrectomy in all the experiments, the adrenals being removed in two stages, one to two weeks apart.

Stewart (1924) has stressed poor technique as a factor in the rapid death of epinephrectomized animals, and discredited much of the early work, on the ground of injury to the animals. In earlier work (Stewart and Rogoff, 1917, 1919) they have shown that sectioning of the splanchnics prevents adrenalin from being secreted into the blood stream. On the other hand, they and Marshall and Davis (1916) found that cats showed no signs of adrenal insufficiency when the splanchnic nerves were out and one adrenal removed. We have found that removal of one gland has no noticeable effect on cats, and in series C the severe handling of one, and the removal of the other gland had no serious effect, provided the blood supply to the cortical tissue left in the animal, was not interfered with to too great an extent. However, we believe that much of the early work can be ruled out on the grounds of faulty technique, especially in those cases in which the animals still retained one adrenal, or died shortly after the removal of both adrenals, without having recovered their full activity for a time.

The chief measure of the efficacy of adrenal removal in cats, is the duration of life after the removal of the second gland, since even a small amount of cortex, if left, is sufficient to maintain life (Biedl 1913; Wislocki and Crowe, 1924). As the value of our work depends to a great extent on the duration of life after complete epinephrectomy, we present an unusually large series of control animals, from which we were able to compute the usual postoperative period in cats deprived of their adrenals. The control operations were performed throughout the entire three years of experimental work, so as to meet the varying conditions of the seasons.

A. Effect of total removal of the adrenals from normal cats. Table 1 shows two series of controls, Z and S. The cats in series S have a slightly shorter survival average than series Z, but this is no doubt due to the fact that they were used for certain blood tests, and the extra handling of the animals and loss of blood would provoke an earlier death.

All of the animals listed in table 1 were not watched during the actual terminal period, but enough of them were, to give a picture of the general occurrences, and these coincide with the observations of previous investigators. In cases in which the animal was found dead, allowance was made in computing the duration of life, this depending on the condition of the body when found.

The removal of the second adrenal at first seems to have no effect. The animal recovers in a short time from the effects of the anesthetic, and for 24 hours seems normal. In many cases (Z 3, 5, 9, 14, 17) the cats drank milk, and in others (Z 1, 4, 18, 19) even ate meat after the operation.

An early symptom is the refusal of food, first solid and then liquid. Most of the animals will drink water until a few hours before death, but not in sufficient quantity to maintain their weight. The weight loss in these control animals averages about 4 per cent of their body weight and

TABLE 1

Life span and weight changes of epinephrectomized cats (control series Z and S)

NUMBER	SEX	DAYS BETWEEN OPERATION I AND II	SURVIVAL IN HOURS AFTER OPERATION II	INITIAL WEIGHT	WEIGHT AT DEATH	WEIGHT CHANGE
		days	*hours*	*grams*	*grams*	*grams*
Z1	♀	7	68	2540	2490	−50
Z5	♀	7	111			
Z9	♀	11	45			
Z14	♀	11	47			
Z15	♀	9	39			
Z17	♀	8	68	2960	2770	−190
Z18	♀	7	68			
Z19	♀	7	51			
Z2	♂	7	70	2720	2610	−110
Z3	♂	10	26			
Z4	♂	7	67			
Z8	♂	7	62			
Z13	♂	25	55			
Z16	♂	7	48	2490	2100	−390
Z20	♂	12	51	2290	2130	−170
S1	♂	9	27	2940	2850	− 90
S2	♂	20	48	3615	3450	−165
S3	♂	18	43	2810	2660	−150
S4	♂	5	55	3110	3095	− 15
S5	♂	8	41	2135	2055	− 80
S6	♂	10	45	2310	2115	−195
S7	♂	6	46	2735	2580	−155
S8	♂	5	59	3410	3280	−130
S9	♂	3	77	2630	2380	−250
S10	♀	13	55	3175	2985	−190
S11	♂	7	52			
S12	♂	5	60			
S13	♂	11	52			
S14	♂	6	50			
S15	♂	8	48			
S16	♂	10	24			
S17	♂	10	50			
S18	♂	4	58			
S19	♂	6	30			
S20	♂	5	48			
S21	♂	7	37			
S22	♂	7	50			
S23	♂	5	72			
S24	♂	13	57			
S25	♂	13	51			
S26	♂	11	75			
S27	♂	13	36			
S28	♂	2	48			

occurs mainly after the first 24 hours following total epinephrectomy, and may be due to lack of food, rather than a result of adrenal insufficiency.

Concurrent with the refusal of food, there is a lack of desire to play with the rest of the animals, the cat preferring to remain quiet. This general lassitude is followed by muscular asthenia, which is first evidenced in the hind legs and later in the fore legs. In spite of this weakness the animal can be aroused to great activity, but sinks to even greater lethargy when the stimulation is removed. The muscular weakness is followed by depression, the animal sinking into a typical position the head on the forepaws and the hind limbs stretched out to the side as shown in figure 2. In the later stages the animal is flat on its side (as in fig. 3,) and either goes in to coma (Z 10, 18, 19) or may have a spasm resembling the "Jacksonian march" with all the extensors coming into play (Z 4, 5, 9). Respiration is rapid until the premortal stage is reached, and then slows down to as low as ten a minute. Some animals emit a sort of cry or moan with each expiration, but this does not always occur. An early symptom is that of lowered temperature of the extremities. This coldness is most easily determined by feeling the ears, which in normal cats are quite warm, but in epinephrectomised animals are cold to touch. The heart goes on beating for a while after respiration ceases, and in a few cases was still beating when the thorax was opened at necropsy. One heart was excised and placed in warm Ringer's solution and kept on beating spasmodically for about an hour after the excision. Other general observations were that the skin becomes gray, dry and wrinkled and loses its elasticity. When the cat is picked up in the usual manner by the back of the neck, the skin remains in a fold for some time. The mucous membranes are dry and lustreless.

Our observations of symptoms following epinephrectomy in cats correspond in most respects to those made by Elliott (1914), Marshall and Davis (1916), and Moore and Purinton (1901). Other investigations have shown that removal of the adrenals causes a rise in the total blood solids (Donath, 1916), doubles the normal amount of urea in the blood (Marshall and Davis, 1915) causes anoxemia and plasma concentration (Gradinscu, 1913) lowers the blood sugar and increases the erythrocyte count (Stewart and Rogoff, 1925).

In view of these findings, and the fact that the symptoms noted agree to a remarkable extent with those of anhydremia as given by Mariott (1923), it would appear that there is a loss of the liquid constituents of the blood. This point will be taken up again in another paper.

Necropsies were performed on all animals, to ascertain whether death was due only to the removal of the adrenal glands. When an examination of the organs showed some pathological cause for death, the case was discarded. Severe infections, when present, were sufficient to rule out a case. Animals that lived longer than usual, were carefully examined for acces-

sory glands, and no suspicious tissue was allowed to escape careful scrutiny and in many cases histological examination.

The average survival time in our control series of 43 cats was 53 hours, the extremes being 24 hours (S 6) and 111 hours (Z 5). This average duration of life after total epinephrectomy serves as the chief criterion in the work that follows, in which we have attempted to determine the survival value of the cortical portion of the adrenal complex following the removal of the medulla.

B. The relative importance of the cortical portion of the adrenal complex for the maintenance of life. Operative procedure: This involves three stages, which in table 2 are referred to as operations 1, 2 and 3.

The first step is the production of a cortical gland by surgical means. One adrenal, preferably the left as that is easier to handle, was exposed by the lumbar approach. Instead of removing the gland, its blood supply was temporarily clamped off with a curved hemostat. It was then opened longitudinally with a sharp razor blade, and the medulla spooned out with a small curette. To make the removal of all the medulla doubly certain, binocular dissecting microscope was used to inspect the gland within the body, and any remaining bits of the grayish medulla, together with a considerable portion of the reticular layer of the cortex were removed. The gland was then sutured with two or three stitches, the clamp on the blood vessels released, and the body wall closed.

A week to ten days later the other adrenal was removed intact, and symptoms observed. If the remaining cortex had not been damaged too severely at the initial operation, the animal recovered, with no signs of adrenal insufficiency.

Three weeks to six months after the second operation, if the animal had developed no unusual symptoms, the surgically produced cortical gland was removed. This third operation is more difficult than the other two, due to the presence of scar tissue and the establishment of a collateral blood supply. In spite of the severity of the operation, the cats survived an average of 4.8 days after the removal of the remaining cortex, the shortest survival being one day and the longest eight days. The fact that the animals in this series survived total epinephrectomy for a longer time than the controls, can be accounted for in the light of Elliott's (1914) work,

Fig. 1. Operated animals in their cages. Three of these animals retain only the cortex of one adrenal.

Fig. 2. Shows the second cat from the right in figure 1 in typical position assumed by epinephrectomized cats shortly before going into coma.

Fig. 3. The same animal in coma, with two cats still retaining the cortex of one gland.

Fig. 4. Two animals, retaining only the cortex of one adrenal gland, shown fighting.

1

2

3

4

and our own data. He found, and our work confirmed his findings, that the survival period is lengthened if three weeks or more are allowed to elapse between operations. In all our cases in this series the wait between the second and third operations was at least three weeks.

An examination of table 2 shows the fate of the animals subjected to the above mentioned operations. The time interval between operations is given and also the survival period after the third operation (cortex

TABLE 2

The survival value of the adrenal cortex (series C)

NUMBER	SEX	DAYS BETWEEN OPERATION I AND II	DAYS BETWEEN OPERATION II AND III	SURVIVAL IN DAYS AFTER III OPERATION	INITIAL WEIGHT OF ANIMAL	WEIGHT CHANGES BETWEEN OPERATION I AND II	WEIGHT CHANGE BETWEEN II AND III	WEIGHT CHANGE BETWEEN III AND DEATH
		days	*days*	*days*	*grams*			*grams*
*C1	♀	11	17	—	2060			−715
C3	♀	8	(12)					
C4	♀	7	(15)					
C5	♀	7	(23)		1200	+10		−520
C6	♀	7	(12)		1150	−140		−130
C7	♀	(21)			930			−40
C8	♀	(27)			1130			−60
C10	♀	7	25	5	4200	−120	+370	−450
C11	♀	7	26	7	3795	−10	+65	−300
C12	♀	7	173	1	2770	+5	+345	−105
†C13	♀	8	23	9+	2385	+125	+180	+20
C15	♀	7	21	6	2030	+40	+20	−180
C16	♀	7	24	2	2505	+25	+55	−275
*C17	♀	7	29	—	3170	−30	−190	—
C18	♀	7	33	3	2875	−30	+565	−255
C19	♀	8	38	8	3840	−50	+760	−680
C20	♀	10	(20)	—	4125	−25		−1115
C21	♀	12	2	—	2375			−235
C22	♀	10	40	2	3005	+180	−65	−120

* C1 and C17 died on the operating table at the third operation.

† C13 was the animal that failed to show symptoms after the removal of the surgically produced cortical gland. On being brought to necropsy, it was found to have an accessory gland of pure cortical tissue.

removal). The weight gains and losses in grams are also given, as well as the original weight of the animal.

One animal (C 1) died on the operating table at the third operation. An examination of the surgically produced cortical gland showed it contained only cortical tissue, thus demonstrating that removal of medulla was possible.

Another animal (C 21) lived only 2 days after the second operation. This may have been due either to the removal of too much cortical material,

or an occlusion of the blood supply at the initial operation. Four kittens (C 3, 4, 5, 6) survived the removal of the medulla of one adrenal and the entire other gland, for a considerable period. Two lived 12 days, one 15 days, and one 23 days. Two more kittens (C 7 and 8) had only the first operation performed on them, and died after 21 and 27 days, while still retaining one gland and the cortex of the other. However, Elliott and Tuckett (1906) have stated that kittens succumb after the removal of a single adrenal. One adult cat (C 20) showed a gradual onset of adrenal symptoms after the removal of the second adrenal. It survived for 20 days. The curetted gland, on sectioning, showed a complete degeneration of the two inner layers of the cortex, and only a small area of the zona glomerulosa was present. This case and that of the four kittens show a gradual production of adrenal symptoms by operative means.

Lacassagne and Samssonow (1923) and Wislocki and Crowe (1924) produced gradual insufficiency symptoms in rabbits and dogs, by implantations of radium in one adrenal and removal of the other. The longest prolongation in their cases was 32 days. Marine and Bauman (1921) produced insufficiency symptoms in rabbits after two weeks, by freezing the glands with ethyl-chloride spray.

Our case C 20 is remarkable in that it recovered from the second operation for a day, as is usual, and then had a period of depression for five days, showing transient adrenal insufficiency symptoms. During this period it drank a great deal of water, but would not take food. It then gradually recovered and was quite active for eight days, but soon began to show signs of muscular weakness and dehydration. Six days after the second onset of symptoms it died in convulsions, which were preceded by a five-hour period of coma. Its weight loss was very great, 1115 grams in 20 days, which was well over 25 per cent of its original weight of 4125 grams.

Ten animals of this series recovered from the second operation and in a very short time could not be distinguished by their actions, from the normal cats in the laboratory, although retaining only the cortex of one adrenal. After an interval of three weeks to six months (see table 2) the surgically produced cortical remnant was removed. Death ensued after an average interval of 4.8 days, the cats showing all the usual symptoms of adrenal insufficiency.

Earlier mention was made of the fact that during the period between the second and third operations, while the animals had only the cortex of one gland, they behaved normally, and ate as much as normal cats, played, fought, and even copulated, as if nothing at all had been done to them. These animals are shown under varying conditions. Figure 1 is a picture of them in their cages in the animal room. Figure 4 was taken out on the roof where the animals were allowed to run. The cage was used to keep the fighting animals within range of the camera. The one on its hind legs

is C 13, the other C 12. Figures 1, 2 and 3 give the story of C 11. In figure 1 it is seen in the second cage from the right, in 2, it is in the typical posture of cats dying of adrenal insufficiency, the head resting on the paws. In 3, it is in coma, and near by are animals C 15 and C 13. In figure 1 it retains the cortex of one gland, and in figures 2 and 3, none. The striking contrast between the animals with and without cortex can be easily seen in figures 2 and 4.

One very notable case (C 13) showed absolutely no symptoms after the third operation and the supposed removal of all its remaining cortical tissue, and after nine days this animal was killed and carefully autopsied. An accessory adrenal was found, the only one in all of the cases listed in this paper. It was found posterior to the kidney and located on the spermatic vein. There were a number of small vessels all leading into the capsule. Figure 5 shows the location of this gland, and also the usual position of the adrenals. This accessory on sectioning, proved to be of pure cortical material, and thus furnishes a natural check on the experimentally produced cortical glands (see fig. 6).

Table 2 gives, in addition to the time intervals between the various operations, and the survival period after the third or final operation, the weight changes that occurred in the animals of this series (C).

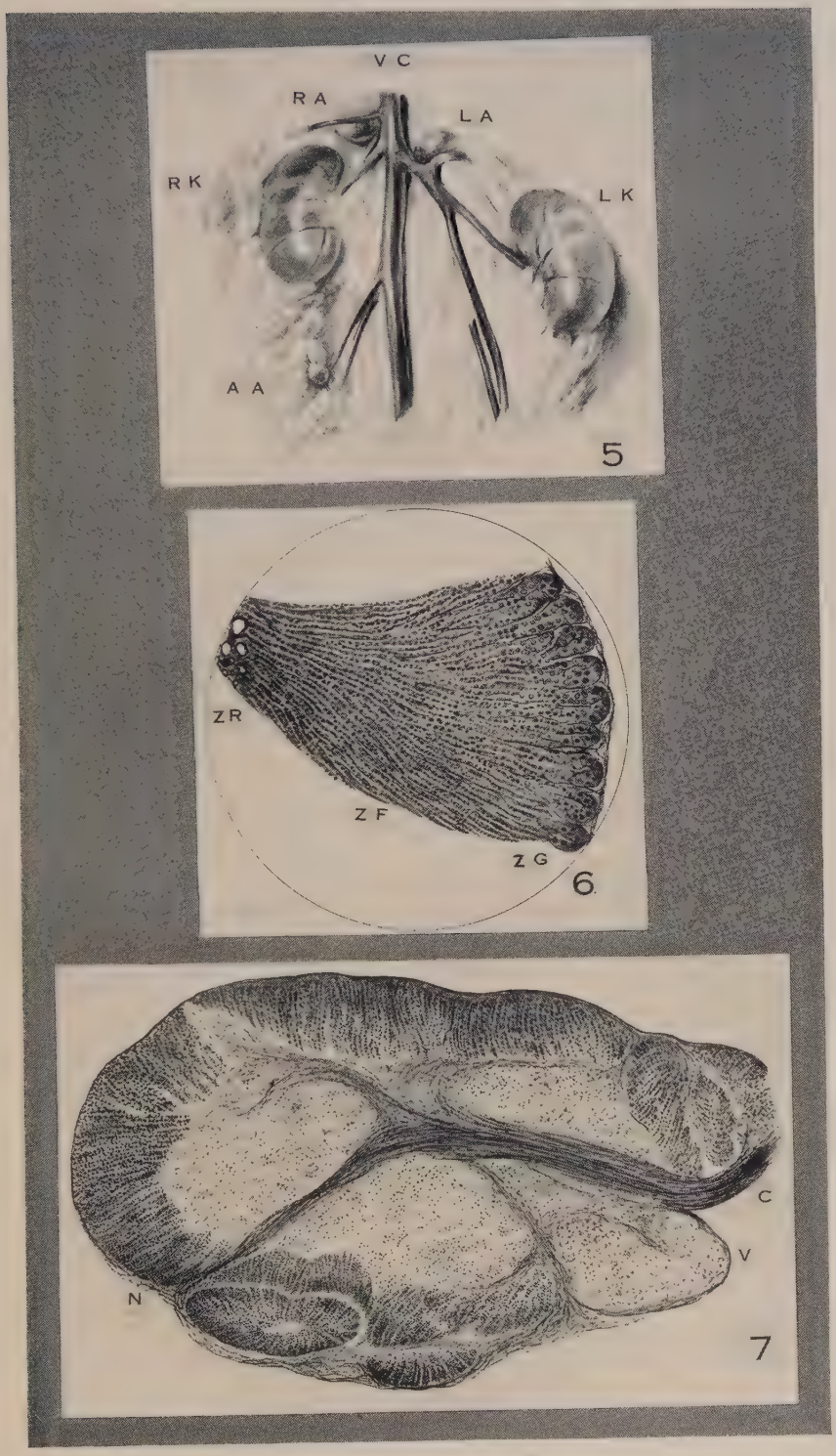
It would appear that the weight loss of animals dying of adrenal insufficiency symptoms depends on the length of time they survive after the removal of all their adrenal tissue, or, in some cases, on the interval between the onset of symptoms and death. One animal, C 19, lost 608 grams in the 8 days that it survived, while C 12, surviving only 1 day, lost 105 grams.

The glands removed at the third operation were preserved and sectioned in order to determine whether or not any medulla had been left behind after the gland had been scraped. For a quick diagnosis in the earlier cases, the frozen section technique was used, and this also proved to be the best in showing the state of the gland and the chromaffine tissue when present. This was obtained by fixing the gland in Muller's fluid-formalin, which stains functioning medulla the characteristic orange-yellow color. The glands were then examined without staining, by sectioning when frozen, and mounting the sections in glycerine. The parts of the gland not

Fig. 5. Drawing showing the position of the accessory cortical gland found in cat C13, and the normal position of the adrenals in relation to the vena cava and the kidneys. *AA*, accessory gland; *RA*, right adrenal; *LA*, left adrenal; *VC*, vena cava; *RK*, right kidney; *LK*, left kidney.

Fig. 6. Section through the accessory gland of pure cortical material. *ZG*, zona glomerulosa; *ZF*, zona fasciculata; *ZR*, zona reticularis.

Fig. 7. Camera lucida drawing of a section through an adrenal from which the medulla had been curretted out, 33 days previously. The central area shows the scar formed after the operation, and some degenerate cortex. *N*, normal cortical tissue; *V*, vacuolated cortical cells; *C*, connective tissue in scar.


V C
R A
L A
R K
L K
A A
5
ZR
ZF
ZG
6
C
V
N
7

used for this determination were later used for making sections by the paraffin method cut at 10μ. Delafield's hematoxylin used alone did not obscure the chromaffin reaction, but other stains were also used, to determine that no chromaffine tissue was present.

Of the ten animals (C 10, 11, 12, 13, 15, 16, 17, 18, 19, 20) that survived the third operation and had the retained cortex sectioned, only two showed medullary tissue distinguishable histolcgically and giving the chromaffine reaction. C 10 showed about 12 cells in a mass of connective tissue, and C 16 a small clump of cells that gave the chromaffine reaction and two smaller groups that were of the same structure, but did not show any chromogen granules. From the above study we determine that eight animals, having only the cortical tissue of one adrenal, lived for three weeks to six months, without showing any signs of adrenal insufficiency, and that on removal of this cortical tissue, they, with one exception, died showing typical symptoms of adrenal insufficiency. The exception, on being brought to autopsy, was found to have an accessory gland, composed of pure cortical material, and with a copious blood supply (figs. 5 and 6).

From this evidence, we believe that the cortical portion of the adrenal complex is the part essential for life in cats, and interference with its blood supply, or its removal from the animal causes the symptoms that have been attributed to the adrenal as a whole. The medulla, at least that within the adrenal complex, is not essential for life or the activity of the animal.

C. The survival value of transplants of the adrenal cortex. Having determined the vital function of the adrenal cortex, the next experiment sets forth an attempt to make functional autografts of adrenal cortex, in the hope that the grafts would maintain the life of animals deprived of their adrenals.

Autoplastic grafts of the endocrine glands have been made using a variety of forms. In mammals, it has been indicated that the gonads, and the parathyroids can be successfully transplanted. A review of grafting experiments of the gonads is given by Lipschutz (1923) and the parathyroids by Morel (1912) and Swingle and Nicholas (1925). Transplantation of the adrenal, however, has not met with much success.

Most, if not all workers, attributed their lack of success to the fact that in their grafts, the medulla rapidly degenerated. In those cases in which success was reported, medullary tissue was always present. It was our belief, from a study of the relative value of the cortex and medulla of the adrenal, that possibly the presence of the medulla interfered with the success of the graft. The following experiments show an attempt to secure the survival of epinephrectomized cats, by means of functioning grafts of the adrenal cortex.

Operative procedure: At the time of the second operation, the remaining adrenal was removed, cut in half longitudinally and the medulla removed.

One-fourth or one-half of the gland was then placed in a pocket prepared for it, either in the rectus abdominis muscle, or beneath the tunica vaginalis of the testis. The latter site was chosen because of Stilling's (1905) success in being able to recognize cortical cells after having been grafted into the testes for three years. A few grafts were tried in the peritoneal cavity, the graft being held in place with sutures.

The peritoneal grafts were made at the time of the first operation, on the assumption that perhaps the time interval between the operations would be enough to allow a blood supply to be established but not long enough for the graft to be resorbed. The grafts into the muscle and testes, being made at the time of the second operation, had to supply an immediate demand. Both types of grafts were equally unsuccessful, in so far as permanent substitution for the whole gland was concerned, but the survival value of the cortex was shown in a prolongation of life, in those cases in which the graft "took."

From an examination of table 3 it can be seen that in five cases the animals derived no benefit from the graft and died within 3 days (B 4, 8, 16, D 4, 5). Of the animals that did live longer than the usual survival period, B 15 lived 4 days, B 2 and 20 lived 5 days. Two of these animals were pregnant and one recently delivered. Of the remaining eleven animals, two lived 6 days, seven 7 days, one 8 days and one 9 days. Counting only the animals that received some benefit from the grafts we get an average survival of $6\frac{1}{2}$ days, which is more than twice as long as that of the control series.

The progress of the insufficiency symptoms of an animal deprived of its adrenals, and with a transplant of cortical material, was much more gradual than that of the controls.

Weight changes in this series were only taken on animals with peritoneal transplants, so may not be significant for the whole series. The changes for the individual animals can be seen in table 3. The transplanted cats lived twice as long, and lost twice as much weight as the control, or completely epinephrectomized animals. However, these animals generally did not eat for 4 or 5 days before death, and starvation may be the factor in the weight losses.

The histological changes of the grafts are not given in tabular form, because they were the same in all cases. The completeness of the degeneration depended on the length of time the graft was in the animal. The sequence of events was: fat formation in the cortical cells; disappearance of cell structure, i.e., nuclei in a vacuolated cytoplasmic mass; necrosis, followed by fibrillar connective tissue invasion.

The results of these experiments, while negative in the production of a functioning graft, are encouraging in that the survival period of animals deprived of their adrenals can be more than doubled by transplants of the

adrenal cortex. The microscopical examination of the transplants showed clearly that no growth had occurred. Therefore, we believe the prolongation to have been due to a resorption of the cortical tissue, and a utilization of the hormones contained in it at the time of implantation. In any event, the fact that grafts of adrenal cortex prolong the life of animals

TABLE 3

The prolongation effect of transplants of the adrenal cortex (series B and D)

NUMBER	SEX	DAYS BETWEEN OPERATION I AND II	SURVIVAL IN DAYS AFTER OPERATION	INITIAL WEIGHT	WEIGHT AT DEATH	WEIGHT CHANGE
a. Grafts in the peritoneal cavity						
				grams	*grams*	*grams*
D2	♀	7	7	3410	3190	−220
D3	♂	7	8	3330	3010	−320
D4	♂	7	2	2490	2200	−290
D5	♀	8	3	2960	2770	−190
b. Grafts in the testes						
B4	♂	25	2	Previous infection		
B14	♂	7	7			
c. Grafts in the rectus abdominis muscle						
B2	♀	7	5	Pregnant		
B3	♀	21	7	Pneumonia		
B5	♀	19	6	Pneumonia		
B6	♀	10	7			
B7	♀	10	9	Slight infection		
B8	♀	11	2	Pneumonia		
B10	♀	8	7	Pregnant		
B12	♀	8	6	Infection		
B13	♀	5	7	Infection		
B15	♀	7	4	Pregnant		
B16	♀	9	2	Pneumonia		
B18	♀	7	7			
B20	♀	14	5	Recently delivered		

Note: During the course of the transplantation experiments feline pneumonia broke out among the animals. This accounts for the incompleteness of the series and probably had some effect on the duration of life after complete epinephrectomy.

deprived of their adrenals, is further indication of the vital function of the cortical portion of the adrenal complex.

Discussion. That the adrenal glands are essential for life has been proved by many investigations. The few exceptional cases on record have been shown to be due to the presence of accessory adrenals in the species

used. Dogs, cats and guinea pigs rarely survive decapsulation, usually dying within a few days of the removal of the glands.

The survival period after epinephrectomy has been used as a criterion in a study of the adrenal complex in the cat. In 43 animals, this averaged 53 hours. The time interval between complete removal of the adrenals and death, depends to some extent upon the time interval between the removal of the first and second adrenal (Elliott, 1914). Stewart and Rogoff (1925) have been able to secure longer survival periods in dogs, than any previous workers. They attribute the rapid onset of symptoms described by previous workers as due to the effects of the operation, rather than to the removal of the adrenals. Undoubtedly the severity of the operation has something to do with the hastening of death after removal of the glands. However, in series C of the present studies, equally severe operations were performed as on the controls, and the animals recovered and were to all appearances normal until the removal of the surgically produced cortical gland. The unilateral operation seems to have no ill effects on the animal, regardless of whether the left or right gland is removed.

The results of the experiments on the survival value of the cortical portion of the adrenal, give further and more complete evidence as to the relative importance of the medulla and the cortex. Cats showed no symptoms of adrenal insufficiency so long as they retained the cortex of one adrenal. Removal of this surgically produced cortical gland induced typical adrenal insufficiency symptoms in the animals, leading to death in a few days. A larger number of animals than have been heretofore reported, underwent the crucial third operation, at which time the cortical remnant was removed.

Evidence for the importance of the cortical portion of the adrenal has been given by Biedl (1913), Wheeler and Vincent (1916) and Houssay and Lewis (1923). This work led to the present studies. Additional evidence for the predominant position of the cortical portion of the adrenal, is offered by the work of Lacassagne and Samssonow (1923), Wislocki and Crowe (1924) and Elman and Rothman (1924).

The evidence for the importance of the medulla offered by Vassale and Zangfroginni (1902) and Vassale (1905) must be disregarded, since their animals died while still retaining one adrenal. They used young cats in their experiments, and Elliott and Tuckett (1906) have shown that kittens succumb after the removal of a single gland.

A. and H. Cristiani (1902) also claim that some medullary tissue is necessary for life, but as they used rats, 60 per cent to 80 per cent of which have accessory cortical glands, their work cannot be taken into account.

The only conclusion possible, from the accumulated data, is that the cortical portion of the adrenal complex is essential for life, and that symp-

toms of adrenal insufficiency are due to removal of the cortex and not the medulla of the gland.

Our experiments on transplantation of the cortex only, were unsuccessful in producing a functional graft, but showed that the presence of the cortical tissue was of some benefit in that it prolonged the life of epinephrectomized cats to more than double the usual survival period. This prolongation, the writer thinks, is due to a resorption of the cortical tissue and its contained hormone, no additional hormones being elaborated by the graft.

It seems a surprising thing that autografts of the cortex do not "take," as autografts of nearly every other tissue are capable of growing in some other part of the same body. The fact that cortical cells persist for some years, but do not sustain life, would indicate that in their case some essential factor was lacking for the proper functioning of the implanted tissue.

SUMMARY AND CONCLUSIONS

1. Unilateral extirpation of the adrenal of cats has no ill effect on the animal.

2. Cats deprived of both adrenals, in two stages with an interval of from 5 to 20 days between operations, will survive on an average, 53 hours; the extremes being 26 and 111 hours.

3. Animals retaining only the cortex of one adrenal with undisturbed blood supply, survive indefinitely, showing that the medullary portion of the adrenal complex is not essential for life.

4. The removal of this surgically produced cortical gland causes the death of the animal in from one to eight days. Histological examination showed that the glands were composed only of cortical tissue. Therefore we conclude that the cortex is the portion of the adrenal complex essential for life in cats.

5. An accessory gland composed of pure cortical material was found to have sustained the life of one epinephrectomized animal.

6. Transplants of the adrenal cortex prolonged life in epinephrectomized cats for an average of $6\frac{1}{2}$ days, but degeneration of the graft eventually caused death.

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AN EXPERIMENTAL STUDY OF THE ADRENAL CORTEX

II. Prolongation of Life after Complete Epinephrectomy

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A. The effect of the thyroid on animals deprived of their adrenals. Earlier investigators have reported changes in the adrenals after thyroidectomy and thyroid feeding, and have shown a correlation of some sort between the two glands. Cannon (1916) found that the adrenal hypertrophied after the thyroid secretion had been augmented by electrical stimulation of the latter gland. Other investigators (R. G. Hoskins, 1910; E. R. Hoskins, 1916; Herring, 1917) observed that thyroid feeding caused the adrenal to hypertrophy in rats. Herring (1916) also found that thyroidectomized adult male rabbits had smaller adrenals than normal. On the other hand, Carlson (1916) stated that thyroidectomy in rabbits causes the adrenal to hypertrophy to two or three times the normal size. This seeming inconsistency can be explained by the work of Gley (1914). He found that thyroidectomy in rabbits produced hypertrophy but not hyperfunction. There was, rather, a fatty degeneration of the cells. This observation is further evidence that the functional activity of an endocrine gland cannot be determined by its weight or size. The relative size of the thyroid in endemic and exophthalmic goitre is another striking demonstration of this fact. In spite of this evidence, some biological statisticians insist that a functional interrelation of endocrine glands can be proved by correlations of weight or size.

Evidence as to the interrelation of the thyroid and the adrenal in metabolism has been offered by Cameron (1923), Marine and Baumann (1922), and Marine, Baumann and Cipra (1925). The latter believe that the adrenal cortex secretes a metabolism depressing substance, since feeding adrenal cortex lowers heat production, and also counteracts the effects of thyroid feeding.

In the following experiments, of which a preliminary report has already been made (Zwemer, 1925) the duration of life, after complete removal of the adrenals, has been taken as a criterion, in a study of the effect of thyroidectomy and thyroid feeding on epinephrectomized animals.

The writer takes the opportunity to express his obligation to Dr. W. W.

Swingle for suggesting this problem and for advice in carrying out the experiments.

Operative procedure. 1. Thyroid absence. In the first four animals listed (series A) the thyroids had been removed about three weeks before the adrenals were removed. The latter were taken out by the lumbar route in two stages.

Eight more animals (series E), were treated as follows: The first time the animal was anesthetized the thyroids and left adrenal were removed. The parathyroids were at the same time dissected out in a medium of sterile Ringer's solution and blood serum and replaced in the neck as grafts, in order to prevent the onset of tetany (Swingle and Nicholas, 1925). At the second operation the right adrenal was removed.

As all of these animals were kept under the same conditions of feeding and living quarters as the controls described in part I of these studies, it can be seen that any variation in the duration of life after the removal of both adrenals would be due to the absence of the thyroids. A study of table 1 shows that the absence of the thyroids tends to delay the onset of adrenal insufficiency symptoms, and to prolong the life of an animal so treated.

The table also gives the interoperative period, weight changes, and the duration of life after thyroid and epinephrectomy. In eleven of the twelve cases there was a considerable prolongation of life. The one exception died of tetany several hours after the second operation, due to failure of the parathyroid graft. It had shown slight tetany symptoms for 9 days previous to the removal of the remaining adrenal and had been relieved by administration of 5 per cent calcium lactate orally.

The longest survivals were 14 days (E 4) and 22 days (A 2), the other cases grading between them, and cat E 7 lived only 3 days. This short lived case was complicated by the fact that it tore open its neck wound. The average survival period including all eleven cases is over eight days (200 hours). This is nearly four times the survival period of epinephrectomized animals still retaining their thyroids, which averaged 53 hours.

2. Thyroid feeding. In order to determine the converse of the above experiments it was necessary to have an additional type of control. These were normal unoperated cats that were fed a definite amount of thyroid per day, merely to see whether it would induce any symptoms in them. Two cats of this series (Y) were fed 4 grains of desiccated thyroid per day for one week, two others were given the same dosage for two weeks. The effects noted were an increased activity and appetite and a decrease in weight. One animal (Y 2) gave birth to a litter of kittens during the course of the feeding experiment, and that accounts for the apparent great decrease in weight, in her case (see table 1).

TABLE 1

The effect of the thyroid on epinephrectomized cats

a. Thyroidectomy (series A and E)

NUMBER	SEX	DAYS BETWEEN OPERATION 1 AND 2	SURVIVAL IN DAYS AFTER SECOND OPERATION	INITIAL WEIGHT	WEIGHT CHANGES BETWEEN OPERATION 1 AND 2	WEIGHT CHANGES BETWEEN OPERATION 2 AND DEATH
		days	*days*	*grams*	*grams*	*grams*
A1	♂	20	6			
A2	♂	19	22			
A3	♀	10	7			
A4	♂	7	6			
E1	♀	12	8	2600	−100	−250
E2	♂	12	8	3000	+100	−510
E3	♂	12	(1 hour)	3100	−660*	
E4	♂	12	14	3200	−90	−310
E5	♂	13	7	3620	−220*	−290
E6	♀	13	5	2860	−50	−280
E7	♀	8	3	2170	0	−170
E8	♀	11	6	2310	+20	−240

b. Thyroid feeding (series F)

NUMBER	SEX	DAYS BETWEEN OPERATION 1 AND 2	SURVIVAL IN HOURS	INITIAL WEIGHT	WEIGHT CHANGES BETWEEN OPERATION 1 AND 2	WEIGHT CHANGES BETWEEN OPERATION 2 AND DEATH	AMOUNT OF THYROID FED
		days	*hours*	*grams*	*grams*	*grams*	*grams*
F1	♀	12	12	2060	−250	−30	24
F2	♂	5		2695	(−105)*		
F3	♀	8	15	2000	−300	−10	24
F4	♂	12	21	2910	−180	−30	36
F5	♀	10	18	2480	−230	−50	32
F6	♂	15	23	3330	−505	−25	52
F7	♂	9		3910	(−200)*		20

c. Normal, unoperated animals fed thyroid as a control (series Y)

NUMBER	SEX	WEIGHT BEFORE FEEDING	WEIGHT AFTER FEEDING	WEIGHT CHANGE	AMOUNT OF DESICCATED THYROID FED, AND DURATION OF EXPERIMENT
		grams	*grams*	*grams*	
Y1	♂	2800	2610	−190	56 grains in 14 days
Y2	♀	2650	1970	−680†	56 grains in 14 days
Y3	♂	3100	2980	−120	28 grains in 7 days
Y4	♀	2710	2550	−160	28 grains in 7 days

* E3 and F2 developed severe tetany and died in tetany convulsions. E5 and F7 showed transient tetany symptoms.

† Cat Y2 gave birth to kittens on the sixth day of the experiment, thus accounting for the increased weight loss.

Having determined that feeding thyroid had no severe effects on unoperated animals in the dosage given, this same daily amount of thyroid was given to cats that had been epinephrectomized. The thyroid was also removed, to obviate any complications that might arise due to the thyroid taking up and holding inactive, part of the thyroid feed. The operative procedure was the same as that given above for the thyroid absence type of experiment, and conditions for the experiments were the same, the only difference between the two series being that in the second series, the cats were fed 4 grains of desiccated thyroid per day between the first and second operations.

Seven animals were used in these feeding experiments (series F, table 1). In every case the animal died within 23 hours after the removal of the remaining adrenal, the average for this series being 18 hours. This is in great contrast to the average survival of 200 hours, of animals having the same glands removed, but not fed thyroid. We would emphasize the fact that epinephrectomized animals without thyroids live, on an average, 200 hours; with their thyroids 53 hours; and when fed thyroid only 18 hours.

This rapid death of the thyroid fed animals cannot be attributed to faulty technique, because the same procedure was followed for all the series of animals, and these animals recovered from the operation and walked around, drank milk and in other ways demonstrated that the operative procedure had not affected them in any way. Necropsies in every case showed nothing unusual.

Of the seven animals used, five survived until the second operation (F 1, 3, 4, 5, 6); one (F 2) died of tetany the fifth day after the first operation; the remaining animal (F 7) died of adrenal insufficiency symptoms although still retaining one gland. This case was complicated by the occurrence of transient tetany on the third and fourth day after thyroidectomy, but it gives an indication, together with an earlier case of a kitten (not listed) deprived of both thyroids and one adrenal and fed thyroid, that in some cases, one adrenal may not be sufficient to counteract the effects of excessive thyroid feeding.

The evidence would tend to show that the thyroid has a marked effect on the duration of life of cats after epinephrectomy. Two possible explanations of this effect will be given in the discussion, but in view of the results of the following experiments involving the oral administration of glucose solution and a study of the weight changes, we believe it to be a dehydrating effect of the administered thyroid.

B. The relief of adrenal insufficiency symptoms by the oral administration of glucose solution. Observations on all the animals dying of adrenal insufficiency, showed that many of the symptoms coincided with those of anhydremia. There is a rapid weight loss; the skin becomes gray, dry and wrinkled, and loses its elasticity (as shown by the dry fold when an animal

is picked up); the mucous membranes become dry and lustreless; the extremities are cold, although the rectal temperature may be high; the respirations in the pre-coma stage, rapid and stertorous, indicating air hunger.

An attempt to relieve this condition by the oral administration of water was unsuccessful. In the two cases attempted, we failed to get prolongation of life and included them in our list of control animals (Z 18 and 19).

Failing to get prolongation with water alone, we next tried the oral administration of glucose solution, hoping the glucose would perform the double feat of assisting in the retention of the water and correcting the marked hypoglycemia which invariably follows bilateral epinephrectomy.

Six animals were used in this experiment. Each of them was given 100 cc. of 5 per cent glucose solution by stomach tube, three times a day. This

TABLE 2

The effect of oral administration of glucose solution on the life of epinephrectomized cats (series G)

NUMBER	SEX	DAYS BETWEEN OPERATION 1 AND 2	SURVIVAL IN DAYS AFTER SECOND OPERATION	INITIAL WEIGHT	WEIGHT CHANGE BETWEEN OPERATION 1 AND 2	WEIGHT CHANGE BETWEEN OPERATION 2 AND DEATH
		days	*days*	*grams*	*grams*	*grams*
G3	♂	8	11	3400	+10	−140
G4	♂	7	6	2200	+20	−390
G5	♂	8	8	2110	+20	−160
G6	♂	7	9	2950	+25	−380
G7	♀	13	8	2550	−10	−170
G8	♂	13	10	2270	+5	−165
G9	♀	10	5	1270	+30	−120

G4 and G9 were not included in computing the average survival as they both developed slight stitch infections, and any infection tends to shorten the survival period of epinephrectomized cats.

was done immediately following complete epinephrectomy, which was done in two stages, the time interval between operations being 7 to 13 days.

The symptoms of adrenal insufficiency appear the fourth day after the second operation, and the onset of symptoms is very gradual as compared with the controls. The average duration of life in these animals, omitting the two having infections, was 9 days, or 220 hours.

If the animals could have been induced to eat, or if food had been given them by stomach tube, they might have survived a much longer time, but in order to be sure that it was the glucose solution that was prolonging life, nothing else was given until the fifth day after the second operation. Then, in four cases (G 3, 4, 5, 6), 1 gram of peptone and 2 cc. of cod liver oil were added to 100 cc. of the glucose solution. This was omitted in cases G 7 and G 8 as it seemed to have no beneficial effect on the animal.

So far as the writer is aware, this is the first successful attempt to prolong the life of animals deprived of their adrenals, by the oral administration of glucose or any other solution.

There are two papers in the literature that indicate that the introduction of liquid into epinephrectomized animals is beneficial. Soddu (1899) reported slight alleviation of symptoms by bleeding dogs and then injecting them intravenously with saline solution. Stewart and Rogoff (1925) found that intravenous injections of Ringer-Locke-glucose solution greatly prolonged the life of dogs deprived of their adrenals. Some of their animals survived as long as 32 days, the intravenous injections being given at intervals of a few days. No symptoms of insufficiency were observed until about 48 hours before death.

Aub, in a very brief abstract (1925), stated that glucose had no effect on the symptoms of adrenal insufficiency in cats. In the same abstract he gives 33 hours as the average survival time for his series of control animals, and claims to have prolonged this to 55 hours, by the administration of extracts of the adrenal cortex. Both of these periods are within the range of our control periods (average 53 hrs.), and for this reason the prolongation seems too short to be significant.

Hartman (1926) reports an average prolongation of life of epinephrectomized animals to 146.6 hours, with a saline extract of the cortex.

Mention has been made of the success of Soddu (1899), Stewart and Rogoff (1925) and the writer (1925) in prolonging the survival period of animals deprived of their adrenals. Corey in this laboratory (in print) has also found that a large variety of salt solutions, when given orally, will greatly prolong the survival period of animals deprived of their adrenals. Some of the effects of cortical extracts mentioned in literature may be due to the introduction of considerable amounts of liquid into the animals dehydrated by epinephrectomy.

The effects of the thyroid, and of the oral administration of glucose solution can also best be explained on the basis of dehydration especially in the light of recent work by Swingle (1926) of this laboratory. The work of Swingle, which is the first complete study of the changes in the blood and urine of epinephrectomized cats, indicates some of the causes back of the dehydration phenomena. A possible explanation of this, and the foregoing experiments with the thyroid, will be taken up in the discussion.

Discussion. The experiments showing the relation of the thyroid to the life of animals deprived of their adrenals shed further light on the rôle of the adrenal cortex in the organism.

In the absence of the thyroid, epinephrectomized cats survived an average of 200 hours, whereas the average survival of the control animals, that still retained their thyroids, was 53 hours. On the other hand, the

feeding of thyroid, thus giving the animal an excess of the hormone, shortens the average survival period after epinephrectomy to 18 hours.

If the rôle of the adrenal cortex is that of a detoxicating agent, as many investigators assume, the explanation of the thyroid effect would be that, in the absence of the thyroid, metabolism is slowed down and fewer toxic metabolites are formed. The feeding of thyroid would have the opposite effect of greatly accelerating metabolism, thus increasing the possible formation of toxic substances.

A more probable explanation is offered in considering the thyroid as a dehydrating agent. Eppinger (1917) found that in edema due to myodegeneration of the heart, or to various types of nephritis, thyroid administration causes a discharge of fluid from the body. On the other hand, after thyroidectomy elimination of fluid is much delayed. Cherfils (1926) reports similar effects after thyroid administration in a number of clinical cases of nephritis. Loeb (1923) thinks it probable that the thyroid substance acts primarily on the intermediary distribution of salts and water, rather than on the kidney. It has long been known that in marked cases of myxedema, due to atrophy or hypofunctioning of the thyroid, rapid loss of water occurs shortly after administering thyroid material.

Since the symptoms of animals dying of adrenal insufficiency coincide in a most striking manner with those of anhydremia, the removal of the thyroid, which is known in certain cases to act as a dehydrating agent, may have delayed the onset of fatal symptoms by slowing down the elimination of water from the organism. Conversely, it is possible that feeding of thyroid substance for a considerable period, caused an early death after removal of the adrenals, due to the rapid elimination of water. The loss in weight of these animals between the first and second operation (table 1) favors this explanation.

The relief of adrenal insufficiency symptoms by oral administration of glucose is striking. The average prolongation was 220 hours. This is over four times the usual survival period of epinephrectomized cats.

Reports of relief of adrenal insufficiency symptoms in dogs, by intravenous injections, have been given by Soddu (1899) and Stewart and Rogoff (1925). Rowntree (1924) of the Mayo Clinic used intravenous injections of glucose to relieve shock occurring in cases of Addison's disease (as much as 600 cc. of a 10 per cent solution).

Recent work by Swingle in this laboratory (in print) indicates that one of the underlying factors back of the dehydration of epinephrectomized animals is an acidosis. He found a marked fall in the pH of the blood of cats from which the adrenals had been removed. With this fall in the pH there is a rise in the inorganic phosphates and an accumulation of organic acids. It is known that dehydration phenomena are associated with acidosis.

SUMMARY AND CONCLUSIONS

1. Feeding of thyroid substance to unoperated normal cats, causes a decrease in the weight of the animal. This is believed to be due to the dehydrating effect of the thyroid, and to the increased metabolism.

2. The removal of the thyroid glands prolongs the life of animals deprived of their adrenals to an average of 200 hours. The absence of the thyroid apparently tends to decrease the elimination of water, and the symptoms of adrenal insufficiency, which strikingly resemble those of anhydremia, are postponed.

3. An excess of thyroid substance, produced by daily feeding of the desiccated gland, during the interval between the removal of the first and second adrenal, hastens the death of epineprectomized animals. The thyroid appears to increase the elimination of water from the organism, thus adding to the dehydration effect following adrenal extirpation. The animals survive on an average only 18 hours after the removal of the second adrenal.

4. The oral administration of a 5 per cent solution of glucose, if given in sufficient quantities, will prolong the life of epinephrectomized cats for an average of 220 hours.

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STUDIES ON THE FUNCTIONAL SIGNIFICANCE OF THE SUPRARENAL CORTEX

I. Blood Changes Following Bilateral Epinephrectomy in Cats[1]

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Numerous investigations have appeared within the last few years concerned with the function of the suprarenal glands. The problem of the functional significance of these structures has been attacked from various angles, but to date the rôle played by the adrenals within the organism remains an unsolved mystery. Despite the sacrifice of many animals, and a vast amount of labor, the cause of death following ablation of these glands is unknown.

The researches of Biedl (1913), Wheeler and Vincent (1917), Wislocki and Crowe (1924), Houssay and Lewis (1923), and Zwemer (1925–26) in this laboratory, show conclusively that the portion of the adrenal which is essential for life is the cortex, since destruction of the medulla is without demonstrable effect, whereas severe injury to or removal of the cortex results in typical symptoms of adrenal insufficiency terminating in death.

Certain changes in the blood of mammals following adrenal removal have been reported from time to time, but so far as the writer is aware no systematic study of the blood chemistry of bilaterally operated animals has been made. It was our original intention to make a thorough study of all of the blood constituents, but as the work progressed certain phases proved especially interesting and worthy of further consideration, and so were studied in detail, thereby necessitating a curtailment of the original program.

Cats were chosen for experimental animals chiefly because they rarely possess accessory adrenals, and bilateral epinephrectomy is usually followed by death within a short period. Zwemer (1926) working in this laboratory, found that cats which have had their adrenals removed at two

[1] The expenses of this investigation were defrayed by a grant from the Bache Fund of the National Academy of Sciences. The writer is indebted to the Biological Laboratory, Cold Spring Harbor, N. Y., for laboratory facilities during the summer of 1926.

stages five to ten days apart rarely live more than sixty or seventy hours. The present writer found that the average survival of forty-nine bilaterally operated cats (interval of seven days between operations) was sixty hours. All of these animals were operated during the fall and winter months. Cats which died within the first eighteen hours were excluded since it is probable that other complicating factors besides adrenal removal may have shortened the survival period. Since this work was begun, the writer and his students have bilaterally epinephrectomized over two hundred animals and have perfected an operative technique which is simple and involves practically no shock to the animal. Only carefully selected cases were employed for blood analysis, and cats which had colds, stitch infections or any symptoms not usually associated with suprarenal insufficiency were excluded.

The writer takes this opportunity of expressing his obligation to Miss Carmen Rothwell of the Pediatrics Laboratory of the Yale Medical School, and to Miss Phyllis Stanley of the Presbyterian Hospital, Newark, N. J., for assistance in carrying out the blood analyses.

The following methods were employed: sodium, Kramer and Tisdall (1921); phosphorus, Briggs' modification of the Bell and Doisy method (1922); calcium, Kramer and Tisdall (1921); non-protein nitrogen, Folin and Wu (1919); total nitrogen, Kjeldahl; blood sugar, Folin and Wu (1919); acetone, Van Slyke (1917). Potassium and magnesium determinations were also made but with such inconstant results that the figures are not included.

The left adrenal gland was first removed from the animal and five to seven days allowed to elapse before the right gland was extirpated. Unilaterally operated cats do not show symptoms of adrenal insufficiency if the remaining gland is normal. Two cats out of two hundred cases under observation at this laboratory died with typical symptoms following removal of one gland. In both cases autopsy revealed a degenerate and atrophic right suprarenal—the degeneration involving both cortex and medulla. However, it has been our experience that unilaterally operated cats remain normal, hence all of our control blood analyses were made on animals with one gland removed. The blood was obtained by cardiac puncture while the animals were under ether (with the exception of blood for sugar determinations) and was obtained a few minutes before the remaining adrenal was removed. The second blood was taken at varying times following the onset of marked adrenal symptoms but without ether. The blood for sugar determinations was obtained by first gentling the animals and quickly drawing blood from the heart before the cats had time to struggle. The symptoms of adrenal insufficiency in cats have been described in detail by Elliott and Tuckett (1906) and Zwemer (1926) so need not be repeated here.

Phosphorus. One of the most striking and consistent changes in the blood constituents following adrenal removal is the rise in the serum phosphorus. In most cases the increase is considerable, especially in the pre-coma stage. The accumulation of phosphorus appears simultaneously with the development of serious symptoms. Animals in coma or prostrate in the pre-coma stage invariably show a sharp increase in blood phosphorus. On the other hand, decapsulated animals which show but slight symptoms of adrenal insufficiency, such as lethargy and beginning weakness, have phosphorus values but little above the normal range. It is possible to use the phosphorus level of the blood as a prognostic sign—high phosphorus invariably indicating that coma and death are but a few hours distant.

The phosphorus values for unilaterally operated cats appear to be somewhat variable, ranging from 4.5 mgm. per 100 cc. of blood to 9.8 mgm. The phosphorus level in the double operated cats ranged from 5.9 mgm. to 16.3 mgm. So far as the writer is aware, no one has reported changes in blood phosphorus following adrenal extirpation. The significance of the increase in phosphorus will be discussed later in connection with changes in kidney function. The increase in phosphorus is not to be regarded as solely a terminal or premortal event, because the increase appears before the extreme symptoms are present. Nor is the phosphorus increase to be accounted for on the basis of increased blood concentration. It is true that the blood of doubly operated cats does show concentration but the following experiment indicates that blood concentration is not responsible.

Cat XY was doubly operated and developed symptoms of adrenal insufficiency such as loss of appetite, weakness of the hind legs, and lethargy. The animal was bled at 5 p.m. and then injected with 100 cc. of Ringer's solution. The following morning at 9 a.m. the cat seemed somewhat brighter and was bled again. The animal finally died in coma at 9 p.m. of the same day. The results of the two blood analyses are shown below.

Before Ringer's solution

Phosphorus	5.8 mgm.
Calcium	9.4 mgm.
Nitrogen	1.15 grams
Protein	7.2 grams
Non-protein nitrogen	41.4 mgm.

After Ringer's solution injected

Phosphorus	6.9 mgm.
Calcium	9.0 mgm.
Nitrogen	0.99 gram
Protein	6.2 grams
Non-protein nitrogen	40.4 mgm.

TABLE 1

Changes in the blood following epinephrectomy

CAT	SEX	UNILATERALLY OPERATED ANIMALS								BILATERALLY OPERATED ANIMALS							
		P	Ca	Na	Protein	Total nitrogen	Non-protein nitrogen	Urea	Symptoms	P	Ca	Na	Protein	Total nitrogen	Non-protein nitrogen	Urea	Symptoms
		mgm.	*mgm.*	*mgm.*	*per cent*	*per cent*	*mgm.*	*mgm.*		*mgm.*	*mgm.*	*mgm.*	*per cent*	*per cent*	*mgm.*	*mgm.*	
1	♂	7.2	9.9	342	8.8	1.41	42.0		None	12.2	9.8	331	9.7	1.49	91		Verging on coma
2	♀	6.1	9.5	339	7.5	1.25	43.0		None	9.2	9.8	328	8.6	1.32	68		Verging on coma
3	♂	4.5	10.2	405	7.5	1.25	44.8		None	5.9	8.6	324	5.7	1.02	97		Very weak. Shock*
4	♀	7.7	9.4	345	7.3	1.22	46.5		None	8.2	9.2	323	7.1	1.22	77		Very weak. Shock*
5	♂	9.5	11.0		8.2	1.31			None	15.2	12.1		8.6	1.37			Coma
6	♂	6.5	10.3		8.2	1.31			None	13.0	11.4		9.2	1.47			Weak
7	♂	9.8	8.4		8.0	1.28			None	15.1	9.7		8.5	1.37			Coma
8	♂	8.4	10.4						None	12.3	12.8						Very weak
9	♂	6.1		312					None	11.6		310					Very weak
10	♂	8.1		335					None	16.3		330					Coma
11	♂	9.2		336					None	13.4		329					Coma
12	♂	6.2	10.5						None	9.4	11.2						Verging on coma
13	♂	4.9					43.5	16	None	8.9					170	122	Coma
14	♀	6.8					44.0	26	None	10.6					76	51	Weak
15	♂						44.0	24	None						64	39	Weak
16	♂						49.0	22	None						91	60	Weak
17	♂				5.8		44.5	19	None				7.1		96	63	Weak
18	♂				6.4		41.0	21	None				7.1		67	40	Weak
19	♂				7.3		42.5	23	None				8.4		89	57	Very weak
20	♂				6.2				None				7.5				Weak

* Animals 3 and 4 died of shock and not from adrenal insufficiency.

A comparison of the two bloods taken before and after Ringer's Solution was injected shows that the degree of blood concentration does not account for the phosphorus rise, because the phosphorus continued to rise despite the fact that the blood had undergone dilution, as indicated by the decrease in protein and total nitrogen. Twelve hours after the second blood was obtained the serum phosphorus had risen from 6.9 mgm. per 100 cc. to 10.4 mgm. per 100 cc. (table 1, animal no. 8).

Calcium and sodium. The changes in the serum calcium following adrenal removal are not of any great significance. In general the calcium rises slightly after the second operation. In view of the fact that the serum phosphorus rises it was considered likely that the calcium would be decreased below the normal level, since several investigators have demonstrated that an inverse ratio exists between the levels of phosphorus and calcium under certain conditions. Barlow and Ellis (1924) cite a single case of an epinephrectomized cat in which the serum calcium rose from 10 mgm. per 100 cc. in the normal animal to 17 mgm. per 100 cc. following double adrenal removal. The blood specimen was taken shortly before death. None of our cases showed increases as great as this. Table 1 shows that the level of the serum calcium of the unilaterally operated cats ranged from 8.4 mgm. to 11 mgm. per 100 cc. of blood, whereas the doubly operated animals showed ranges from 8.6 mgm. to 12.8 mgm. per 100 cc. The slight increase in the serum calcium can best be accounted for as being due to increased blood concentrations.

Only a few sodium determinations were made since it soon became evident that this substance remained practically unchanged after adrenal extirpation. In general a slight drop in the serum sodium occurred (table 1) but the decrease is not of sufficient magnitude to warrant serious consideration. We did not anticipate any marked change in the sodium content of the blood since the level of this substance is not known to vary greatly in any pathological condition (table 1).

Non-protein nitrogen. The non-protein nitrogen always shows a sharp rise following double epinephrectomy, and at times the increase may be very great (table 1). The average value for non-protein nitrogen in unilaterally operated cats is between 42 and 47 mgm. per 100 cc. of blood, whereas the average amount found in the blood of double operated animals is about 91 mgm. Like the serum phosphorus, the non-protein nitrogen is always highest when the animals is in or verging on coma. The increase becomes apparent within the first twenty-four hours after operation and slowly increases until the animal enters the pre-coma stage when a sharp rise occurs. The highest figure for non-protein nitrogen recorded in our data is 170 mgm. per 100 cc. (cat 13, table 1). With the exception of this animal, the data presented in table 1 show that none of our figures are excessively high. The non-protein nitrogen values

for uremia, for instance, are far beyond anything encountered in bilaterally operated cats. In such cases the non-protein nitrogen may be as high as 360 to 400 mgm. per 100 cc. of blood. On the other hand true cases of uremia may show values approximating those of our bilaterally operated cats. It should be recalled that for the first eighteen hours following bilateral epinephrectomy, cats remain practically normal, and that the entire train of symptoms leading to death from adrenal insufficiency occurs within the next twenty to thirty hours. Apparently sufficient hormone from the adrenal cortex circulates in the blood to maintain the animal in a fairly normal condition during the first eighteen hours after double operation. Considering the short length of time between the second operation and death (about 60 hours) the increase in non-protein nitrogen is rapid.

Urea. The data on urea are not so extensive as could be desired, since but seven cases were studied (table 1). However, these cases are sufficient to show that the blood urea rises rapidly following double adrenal removal. This is of course to be expected considering the consistent rise in non-protein nitrogen which occurs.

There are other cases in the literature where increases have been reported in cases of adrenal insufficiency. Marshall and Davis (1916) reported that an increased concentration of urea occurs in the blood of cats following adrenal extirpation. They state that in cats which survive for several days after removal of both glands, the blood urea rises shortly after the second operation and attains a concentration about double the normal value. It remains at this level until about twenty-four hours before death, when a further steady rise occurs.

Marshall and Davis also report that analysis of the tissues of two dogs which died as a result of adrenal extirpation showed the presence of about five times the normal amount of urea.

Porak and Chabanier (1908) report finding an increase in the urea content of the blood of epinephrectomized rabbits eight to ten hours following adrenal removal.

It is interesting to observe that Rowntree (1925) in the course of an investigation of Addison's disease reports that several of his cases had high blood urea. He says: "Renal function studies were made in twelve cases. The blood urea was 30 mgm. or more for each 100 cc. in ten, 50 mgm. or more in four, and between 90 and 100 mgm. in two. Each of the last two patients appeared to be in extremis at the time the determinations were made, but the increase in blood urea remained unexplained. In one, a fatal case, the increase in urea persisted, but in the other patient it dropped quickly as the patient improved and it became normal within a week."

The evidence seems quite clear that the urea content of the blood rises following lesions or removal of the adrenal glands.

Protein and total nitrogen. The total nitrogen of the serum shows an increase in bilaterally operated cats. Table 1 shows the total nitrogen determinations for seven animals. It will be noted that cats 3 and 4 do not show an increase following the second operation. In fact the total nitrogen of cat 3 dropped from 1.25 grams per 100 cc. to 1.02 grams per 100 cc., and cat 4 remained unchanged. The protocols of both of these animals reveal the interesting fact that they died within twenty-four hours following removal of the remaining adrenal. All of the other cases reported lived considerably longer. Cat 3 survived the second operation twenty-four hours and forty minutes, and cat 4 lived but eighteen hours. The short survival period following the second operation renders these two cases doubtful. This probability receives support from a study of the other blood constituents. The serum protein and total nitrogen of cat 3 show decided decreases whereas in all other cases dying from typical symptoms of adrenal insufficiency, protein and nitrogen increase. Non-protein nitrogen increased in both cases however. These two animals are included in table 1 because they offer excellent confirmatory evidence for the view that all animals dying within twenty-four hours after removal of the second adrenal should be discarded as unsatisfactory cases since other factors than adrenal insufficiency probably caused death.

With the exception of the two animals mentioned the percent of serum protein rises after adrenal removal although the increase is not so great as we would expect considering the apparent increase in blood concentration.

Blood concentration. The blood of double operated cats shows considerable evidence of water loss. It is concentrated, does not flow freely into the syringe, clots more rapidly than the blood of unilaterally operated animals and yields smaller amounts of serum. Barlow and Ellis (1924) found that the blood coagulation time in 17 doubly operated cats was consistently shorter than in normal or unilaterally operated animals, and became progressively shorter as the death point was approached.

Blood concentration in our cases is indicated by the increase in serum protein (table 1). This increase appears to be due to a decrease in the liquid portion of the blood rather than to an actual increase in protein. It is the writer's impression that the concentration of the blood of bilaterally operated cats presenting marked symptoms is greater than the figures for serum protein indicate. Hemoglobin and cell volume determinations will be made in the near future to test this point.

Stewart and Rogoff (1925) made blood examinations of double operated dogs and state: "It is common to find towards the end of the survival period, perhaps only on the day before death or on the day of death, that

the relative volume of the serum dropped sharply. This was not infrequently accompanied by a diminution in the conductivity of the serum." These authors, however, caution against laying too much stress upon the terminal concentration of the blood. They report increases in hemoglobin in double operated dogs.

The evidence seems to indicate that a marked concentration of the blood occurs in bilaterally epinephrectomized animals about eighteen to twenty hours before death. Diluting the blood by injections of saline or glucose revives cats verging on coma but the effect lasts only a few hours. We have repeatedly injected cats in coma or verging on coma with gum-acacia in saline solution to increase the blood volume and raise its osmotic pressure. Such injections occasionally bring about a striking amelioration of symptoms but the effect is temporary and lasts but an hour or two.

TABLE 2

Blood sugar changes in normal and bilaterally operated cats

	NORMAL	OPERATED	SYMPTOMS
	mgm. per 100 cc.	*mgm. per 100 cc.*	
Cat 1	100	40	Coma
Cat 2	106	60	Prostrate
Cat 3	111	60	Prostrate
Cat 4	100	66	Prostrate
Cat 5	112	40	Coma
Cat 6	110	50	Coma
Cat 7	114	66	Prostrate
Cat 8	112	55	Coma
Cat 9	117	40	Coma
Cat 10	115	60	Very weak
Cat 11	112	55	Very weak
Cat 12	120	45	Prostrate

Blood sugar. It has long been known that the blood sugar of epinephrectomized animals shows a marked drop, and the same thing has been repeatedly observed in cases of Addison's disease. The determinations for blood sugar given here were made on a different set of animals than those employed for the other blood analyses. This was necessary because a normal blood sugar determination cannot be obtained from an anesthetized animal. The control blood was taken from unoperated cats. The following table shows blood sugar changes in twelve animals before and after bilateral epinephrectomy.

In accordance with the results of other investigators, table 2 shows that a striking fall in blood sugar occurs following adrenal extirpation. The normal blood sugar for cats is 105 to 118 mgm. per 100 cc. The drop begins about twenty hours after recovery from the anesthetic and con-

tinues slowly until death supervenes. One feature about the blood sugar decrease proved especially interesting—namely, that the onset of the symptoms of lethargy and muscular asthenia coincided with the drop in blood sugar. It was repeatedly noted that cats which were beginning to show muscular weakness and giddiness when walking, had blood sugars between 75 to 80 mgm. per 100 cc. of blood, and as the sugar decreased the asthenia became more marked until finally when coma developed, the blood sugar had fallen to between 40 to 55 mgm. per 100 cc.

These observations led to the belief that the muscular asthenia and progressive fall in body temperature which are practically invariably present as part of the train of symptoms of adrenal insufficiency in cats are due in part at least to the decrease in blood sugar. It is not claimed that the fall in blood sugar is the primary cause of adrenal death—far from it—for we have repeatedly injected double operated cats exhibiting marked symptoms with glucose, and observed that death can and does occur with high blood sugar. However, the hypoglycemia is of sufficient degree to

TABLE 3

Blood sugar of insulin injected cats

	NORMAL SUGAR	UNITS INSULIN	HOURS	SYMPTOMS	BLOOD SUGAR
	mgm.				*mgm.*
Cat 44	115	15	4	Coma	70
Cat 49	112	15	5	Coma	50
Cat 50	110	15	5	Coma	45
Cat 51	114	15	4	Coma	60
Cat 54	125	15	5	Coma	50

account for the asthenia and fall in body temperature. The following experiment bears upon this point.

A series of five cats was injected with insulin in order to produce a condition of profound hypoglycemia, and the symptoms compared with those following adrenal removal. In both conditions the onset of muscular weakness and giddiness occurred when the blood sugar had fallen to below 90 mgm. per 100 cc. of blood. The animals became unconscious and developed convulsions when the blood sugar fell below 60. Death occurred when the sugar of the blood reached the level of 55 to 40 mgm. In both "insulin shocked" cats and animals showing marked symptoms of adrenal insufficiency, the body temperature fell and the blood showed signs of concentration. Table 3 shows the blood sugar level at which normal cats (half grown) reach the convulsive stage when injected with insulin.

It was stated previously that the hypoglycemia itself cannot account for death following adrenal removal, because bilaterally operated cats

injected with glucose invariably die sooner or later despite the high blood sugar. However, cats in deep coma or verging on coma can be revived to a striking degree by injections of glucose. Within an hour following subcutaneous injections of 100 cc. of 10 per cent glucose plus 10 units of insulin an epinephrectomized cat in coma can be revived to such an extent that it will sit up or even walk about and seem on the road to recovery. Such animals show a high concentration of blood sugar, but despite this generally die in convulsions within several hours. Hence it is evident that the low blood sugar alone is not sufficient to account for the death of epinephrectomized cats, although all the symptoms of muscular weakness and giddiness can be attributed to this cause, because in insulin injected animals weakness, giddiness and convulsions appear when the blood sugar reaches the same level at which similar symptoms occur in operated animals. If an insulin injected cat is prostrate and on the verge of coma when the blood sugar drops to 50 mgm. per 100 cc. of blood, and we find the same symptoms and same blood sugar level in epinephrectomized cats, it seems fair to draw the inference that in both cases the symptoms are probably due to the hypoglycemia. If this be granted the cause of death of the double operated animals still remains unexplained, because glucose injections exert only a temporary effect. What other factor or factors are responsible? An attempt to answer this question will be made following presentation of certain other data.

The urine. During the course of the work it was noted that on the day when death occurred or on the day previous, the cats passed little or no urine. Some animals showed complete suppression, others were able to pass small quantities up until a few hours of death. Comparison of the total amount of urine passed by unilateral and bilaterally operated animals shows that after the second adrenal is removed the quantity of urine is much diminished.

Cat 93. Right adrenal removed April 13, 3 p.m. Left gland removed April 15.
April 14 passed 57 cc. urine containing 0.35 mgm. acetone per cubic centimeter.
April 15 passed 44 cc. urine containing 0.27 mgm. acetone per cubic centimeter.
April 16 passed 22 cc. urine containing 0.18 mgm. acetone per cubic centimeter.
April 17 passed 6 cc. urine containing 0.33 mgm. acetone per cubic centimeter.
Animal died in convulsions at 7 p.m. April 17. No urine in bladder.
Cat 94. Right adrenal removed April 13. Left gland extirpated April 15.
April 14 passed 62 cc. urine containing 0.09 mgm. acetone per cubic centimeter.
April 15 passed 56 cc. urine containing 0.28 mgm. acetone per cubic centimeter.
April 16 passed 24 cc. urine containing 0.24 mgm. acetone per cubic centimeter.
April 17 passed 16 cc. urine containing 0.25 mgm. acetone per cubic centimeter.
Animal died in convulsions at 5 p.m. April 17. No urine in bladder.

It is obvious that the output of urine is decreased in double operated cats. This can be partially accounted for on the basis of lowered blood pressure, since blood pressure falls in double operated animals. How-

ever, it has been shown by Marshall and Davis (1916) that the excretory function of the kidney of bilaterally epinephrectomized cats is diminished even when a normal blood pressure is maintained. They observed that the ability of the kidney to excrete certain dyes was considerably lessened after adrenal removal.

The data show that the ability of the kidney to excrete acetone remains normal up until the time of death. Albumin occurs in the urine of the double operated cats showing marked symptoms, but the concentration is not great.

The kidneys. Because of the retention of phosphorus and non-protein nitrogen in the blood, and the presence of albumin in the urine, the kidneys were examined for pathological changes. The procedure was as follows: The left adrenal and left kidney were removed and the kidney cut into thin pieces and fixed in Zenker's fluid. Five to seven days later the right adrenal was removed with especial precautions not to injure the kidney. When the cat passed into coma about sixty hours later, it was killed and the remaining kidney preserved, sectioned and compared with the normal left kidney. Delafield's hematoxylin, and eosin, were used for staining the tissue.

Macroscopic examination showed little or no change in the appearance of the kidney of the double operated animal beyond a slight congestion. Histological examination revealed several slight pathological changes such as small hemorrhagic areas throughout the cortex and cloudy swelling of the tubule cells. A detailed account of the pathological changes found in the kidneys of the double operated cats will be reported later.

Discussion. The chief findings resulting from this study of the blood chemistry of bilaterally epinephrectomized cats are: increase in the serum phosphorus, accumulation of urea and non-protein nitrogen, decrease in blood sugar, concentration of the blood, diminished urine output, and presence of albumin.

A study of the acid-base equilibrium of bilaterally epinephrectomized cats, carried on simultaneously with the present work (Swingle and Eisenman, 1926) revealed several significant facts when taken in conjunction with the results of this study. There is always a fall in pH, carbon-dioxide tension, serum bicarbonate, and total acid; indications of an uncompensated, non-volatile acidosis which apparently is due to an increase of phosphoric and organic acids.

The data indicate that death following removal of the adrenal glands may possibly be due to either of two pathological changes in the organism, neither of which are necessarily connected, but either of which is of sufficient magnitude to result in death if left uncorrected. The hypoglycemia which

results from adrenal removal is, in cats, severe enough to reduce the blood sugar within sixty hours to the level at which cats become unconscious and develop convulsions. The sugar level can be raised by glucose injections, yet the animals die eventually. Hence it is obvious that the hypoglycemia is but one factor responsible for death. The second factor is the failure of the kidney to excrete acid phosphate in sufficient quantities, with the result that an acid intoxication slowly develops which eventually leads to death. It is obvious, on the basis of the viewpoint expressed here, why epinephrectomized animals eventually die despite the fact that the hypoglycemia has been corrected by glucose injections. The injections relieve the blood sugar, but do not prevent the piling up of phosphorus and organic acids in the blood, and the resulting acidosis remains uncorrected.

The type of acidosis which develops following adrenal removal is of a similar nature to the acid intoxication characteristic of the terminal stage of certain types of nephritis. In both conditions failure of the kidney to properly eliminate acids seems to be the cause of the acidosis. The data indicate that the adrenal cortex supplies some hormone necessary for the maintenance of normal kidney function, and also that this secretion of the cortex probably acts as a brake or check upon the secretory activity of the islets of Langerhans.

SUMMARY

1. The blood of unilaterally and bilaterally epinephrectomized cats was analyzed for phosphorus, calcium, sodium, proteins, total nitrogen, non-protein nitrogen, urea and sugar. Significant increases in phosphorus, protein, non-protein nitrogen and urea were found in the double operated animals. The blood sugar decreases to the level at which convulsions occur in insulin injected cats.

2. The blood becomes concentrated, thus accounting for the rise in protein and total nitrogen.

3. The urine output is diminished and albumin appears.

4. Slight pathological changes were found in the kidneys.

5. The suggestion is made that death following adrenal removal may possibly be due to either of two causes: hypoglycemia and acid intoxication, since both phenomena are invariably associated with adrenal insufficiency. The hypoglycemia can be corrected by glucose injection but the animal eventually dies with symptoms of acid intoxication.

6. The acidosis appears to be due to failure of the kidney to properly eliminate acids, and is of the same general nature as that occurring as a result of certain types of chronic nephritis.

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STUDIES ON THE FUNCTIONAL SIGNIFICANCE OF THE SUPRARENAL CORTEX

II. The Acid-Base Equilibrium of Epinephrectomized Cats[1]

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Previous work by the senior writer and his students (Zwemer, 1924, 1925; Corey, 1926; Swingle, 1926) has shown conclusively that the part of the suprarenal complex in mammals that is essential for life is the cortex. The medullary portion can be removed without ill effects resulting, but if the cortex is destroyed or badly damaged, the animal exhibits typical adrenal insufficiency symptoms, and dies within a few days. The earlier work of Biedl (1903), Wheeler and Vincent (1917), Houssay and Lewis (1923), Wislocki and Crowe (1924), likewise indicated the great importance of the cortex for life. The functional significance of this portion of the suprarenal is at present unknown.

Because of certain striking changes in the blood which invariably follow adrenal (cortex) extirpation in mammals (Swingle, 1926) and the fact that the animals present unusual changes in the respiration some hours preceding coma and death, changes indicative of marked air hunger, it seemed probable that acid intoxication was one of the train of symptoms leading to death from epinephrectomy. With the idea of testing this hypothesis, and gaining some insight into the nature of the acidosis, if such existed, the coöperation of the junior author was enlisted.

So far as the writers are aware, no one has attempted a complete and thorough investigation of the chemical and physical changes in the blood following suprarenal extirpation. A few scattered investigations have appeared dealing with the changes in one or other constituent of the blood, but scope of inquiry has been too limited. The present report does not claim to be a complete picture of the blood changes following adrenal removal in cats. We were unfortunate in our choice of experimental animals, since the cat is a difficult animal to bleed repeatedly without serious struggling. On the other hand, we have on hand in the Osborn Zo-

[1] The expenses of this investigation were defrayed by a grant from the Bache Fund of the National Academy of Sciences.

ological Laboratory, the protocols of over two hundred completely epinephrectomized cats, with which to compare those of the present experiments.

METHODS AND PROCEDURE. Arterial or venous blood was drawn from the heart into a Luer syringe containing albolene and transferred without exposure to air into centrifuge tubes containing oil. The tubes were stoppered and the blood was immediately centrifuged. The serum was then transferred without exposure to air into a sampling bulb containing mercury, whence samples could be withdrawn for analysis. The technique used for the treatment of the blood has been described in detail by Austin, Cullen et al. (1922).

Serum proteins were determined by analyzing $\frac{2}{5}$ to $\frac{7}{12}$ cc. of serum (diluted) by the macro-Kjeldahl method. From this total nitrogen value, an average non-protein nitrogen figure was subtracted. The result was multiplied by 6.25 to convert it to protein. The average normal non-protein nitrogen value was obtained from a series of ten analyses of normal cats' whole blood. The values ranged from 38 to 60 and gave a mean of 47 mgm. per hundred cc. blood. The average non-protein nitrogen value for operated cats was obtained from eight determinations of whole blood non-protein nitrogen from operated cats. The values ranged from 41 to 170, with an average of 83 per hundred cc. of blood. Such average corrections are only approximations. The non-protein nitrogen analyses were done on whole blood and on different animals from those used in this study. Whole blood non-protein nitrogen tends to be higher than serum non-protein nitrogen. Furthermore, non-protein nitrogen values for cats seem to be extremely variable. However, sufficient material was never obtained to accomplish the determinations on this series of cats, and, therefore, our protein values are not entirely satisfactory, but they serve to give an idea of concentration changes. The error in the pre-operative determinations probably does not exceed 0.1 per cent; that in the post-operative may be as high as 0.5 per cent of protein.

Serum carbon-dioxide content determinations were made on 1 cc. samples according to the Van Slyke (1924) technique in the constant volume apparatus.

pH determinations were made by the gasometric method described in a previous communication by one of the writers (Peters, Bulger and Eisenman, 1926).

Serum chloride was determined by the Van Slyke (1923) method.

Inorganic phosphorus was determined by the Benedict-Theis (1925) method.

Total base was determined by a slight modification of the Stadie-Fiske (1925) method.

CALCULATIONS. The acid values of protein, bicarbonate, chloride and phosphorus were calculated as previously described (Peters et al., 1926).

Total acid was taken as the sum of all the determined acids.

Organic acid was calculated by subtracting total acid from total base, and, of course, includes sulphate ion.

EXPERIMENTAL. *Normals.* As normal animals, cats were used that had completely recovered from unilateral adrenalectomy. Such animals are perfectly normal and experience with over two hundred cats with one adrenal removed has convinced us that one adrenal is amply sufficient to maintain a cat in normal condition. The blood serum was analyzed for protein, carbon-dioxide content, sodium chloride, phosphorus, pH, and total base. The data are given in table 1. Eleven cats were bled. Eight were bled while under urethane anesthesia; three cats that gave external

TABLE 1

Unilaterally operated cats

CASE	NATURE OF BLOOD SPECIMEN	URETHANE ANESTHESIA	STRUGGLING	SERUM PROTEIN	SERUM PROTEIN	pH	CARBON DIOXIDE TENSION	SERUM BICARBONATE	SERUM CHLORIDE	SERUM PHOSPHORUS	TOTAL ACID	TOTAL BASE	ORGANIC ACID
				per cent	*mM.*		*mm. Hg.*	*mM.*	*mM.*	*mM.*	*mM.*	*mM.*	*mM.*
ZI	Venous	+	0	5.89	10.8	7.48	27.8	21.3	109.6	3.5	145.1	154.1	9.0
ZN	Arterial	+	0	6.40	10.9	7.30	35.2	18.0	115.9	4.0	148.7	161.3	12.6
XC	Venous	0	0	7.32	12.3	7.27	42.7	19.9	113.2	3.8	149.2	163.5	14.3
V	Venous	+	0	6.91	11.6	7.26	33.4	15.5	115.6	2.8	145.5	154.4	8.9
ZM	Venous	0	*	6.28	10.7	7.31	37.0	19.3	118.5	5.6	154.1	175.2	21.1
ZX	Venous	0	0	5.97	10.1	7.29	33.1	16.2	111.3	3.6	141.3	162.6	21.3
SA	Venous	0	0	6.16	10.5	7.31	38.0	19.7	109.4	5.2	144.8	173.1	28.3
SB	Arterial	+	0	6.46	11.5	7.41	33.0	21.6	106.8	3.5	143.3	186.4	43.1
ZK	Arterial	+	0	6.18	10.7	7.35	28.3	15.8	111.8	3.6	141.9	156.7	14.8
ZF	Arterial	+	0	7.78	13.6	7.37	31.0	18.4	103.1	2.5	137.6	146.0	8.4
ZE	Arterial	+	0	7.53	13.2	7.37	26.7	15.8	109.6	3.0	141.5	157.5	16.0

* Slight.

evidence of slight or no struggling were bled without anesthesia. The variations in the individual constituents were so considerable that it was impossible to obtain satisfactory average normal values. If the three specimens obtained without anesthesia are excepted, more concordance in values is observed. The three excepted specimens show high base and high organic acid and the pH is relatively lower than that observed in specimens obtained under anesthesia. These facts, we believe, offer internal evidence of muscular activity on the part of the animals, despite lack of external evidence, as the results are comparable to those of exercise in normal human individuals.

Of the anesthetized animals, SB shows high base and high organic acid. Inasmuch as the total base is extremely high—in fact greater than any

other "normal" total base value recorded, it may be assumed that the analytical value for total base and the consequent calculated value for organic acid are incorrect. The second blood, i.e., that following extirpation of the remaining adrenal was not obtained, the animal dying during the night.

Animals after double adrenalectomy. After the second adrenal had been removed, blood samples were obtained from animals exhibiting symptoms of varying severity. The serum was analyzed for the same constituents as that of the normals or unilaterally operated animals. The results are given in table 2. Certain differences from the normals are grossly evident:

(1) The serum bicarbonate content always drops.
(2) The tension of carbon-dioxide always drops.
(3) There is always a fall in pH.
(4) Proteins rise in all but one case (SA).
(5) There is usually a marked rise in phosphorus, which may become very high when the animals pass into coma (Swingle, 1926).
(6) Total acid always diminishes.

Certain values show variations:

(1) Changes of chloride concentration are variable.
(2) Total base shows a striking drop in three cases. (ZM, ZS and SA.)
(3) Organic acid is not consistently affected, although it tends to be higher in the operated animals.

Discussion. The drop in total acid is almost completely at the expense of bicarbonate. The fall in bicarbonate concentration and carbon-dioxide tension cannot be due to over-ventilation, because the latter would result in an increase of pH. Dyspnea and hyperpnea are invariably present shortly before the animals pass into coma and is one of the striking symptoms of adrenal insufficiency in the preterminal stages. (See protocols.) In these experiments, pH has fallen, indicating that the bicarbonate reduction is due to a non-volatile acidosis for which the respiratory mechanism has not compensated. Chloride concentration is not consistently or significantly altered, and, therefore, cannot be the cause of the carbon-dioxide change. Nor can increase in protein concentration be responsible, because it is offset by the decreased base-binding power of the proteins at the lower pH. The fall in bicarbonate can not be occasioned to any considerable extent by the rise in phosphorus, which although marked, never exceeds 2 millimols, while the carbon-dioxide falls from 6 to 10 millimols.

The increase in organic acid is the one change of sufficient magnitude to be responsible for the bicarbonate fall. In considering the possible causes for the rise of organic acid, ketosis cannot be excluded, since some of the

TABLE 2

Cats after bilateral epinephrectomy

CASE	NATURE OF BLOOD SPECIMEN	URETHANE ANESTHESIA	SYMPTOMS	PROTEIN	SERUM PROTEIN	pH	CO_2 TENSION MERCURY	SERUM BICARBONATE	SERUM CHLORIDE	SERUM PHOSPHORUS	TOTAL ACID	TOTAL BASE	ORGANIC ACID
				per cent	*mM.*		*mm.*	*mM.*	*mM.*	*mM.*	*mM.*	*mM.*	*mM.*
ZI	Arterial	0	Moderate	7.14	11.6	7.19	24.5	9.7	115.9	5.0	142.2	158.9	16.7
ZN	Venous	0	Coma, serious	7.10	11.3	7.14	30.5	10.5	112.8	6.2	140.8		
SC	Arterial and venous	0	No coma	8.42	13.2	7.11	30.7	10.1	116.2	4.8	144.3	164.4	20.1
V	Venous	0	Blood drawn under artificial respiration	7.34					114.4	5.2		155.9	
ZM	Arterial and venous	0	Coma, difficult bleeding						116.1	6.3		155.0	
ZX	Arterial	0	Mild	6.51	10.9	7.27	21.5	10.0	106.0	3.6	130.6	141.5	10.9
SA	Venous	0	Mild	6.11	10.2	7.26	25.5	11.7	111.1	5.2	138.3	160.0	21.7
ZO	Venous	0	Convulsions	6.97	11.0	7.13	31.7	10.9	108.2	6.0	136.1	154.8	18.7

animals received little or no food after the second operation.[2] In the two of the three cases where values for organic acid were obtained, the animal exhibited only moderate symptoms. The rise of organic acid in these cases may well be due, as in the case of the normals, to muscular activity, as no anesthetic was employed. In the single case with severe post-operative symptoms from which a value for organic acid was obtained, the animal unfortunately passed into convulsions, while bleeding was in progress. The high organic acid in this instance is, therefore, of especially doubtful significance.

In these post-operated animals, high organic acid is not, as in the normals, associated with high total base. The blood from these animals gives apparent evidence of concentration. It is viscous and hard to draw and relatively little serum is obtainable from the whole blood. If such concentration is due to dehydration, and such appears to be the case as shown by other work (Swingle, 1926), the reason for the low total base is forthcoming. Dehydration is usually associated with a loss of total base. The usual associated loss of chloride was, however, not observed in our operated animals.

Serum protein is usually used as a measure of blood concentration. Although a slight rise in proteins is observed in the post-operative specimens, this small increase does not correspond to the apparent concentration of the blood. Dehydration and blood concentration in these cases are possibly associated with an actual loss of protein from the serum, due to a changed permeability of the blood vessel wall. The discrepancy can hardly be referable to the errors in protein determinations discussed above.

If the values for the different components are corrected for inspissation of the blood in order to compare the actual amounts rather than the concentrations of these constituents in the blood before and after operation, the conclusions are not altered because the blood volume changes are so small.

The differences between the values for arterial and venous blood are so small that the results are not significantly affected if they are used interchangeably.

It is to be regretted that the data are so incomplete. More extensive experiments are necessary before any definite conclusions can be drawn as to the nature of the acidosis following complete extirpation of the adrenals. There can be no question but that a marked acidosis is one of the cardinal symptoms of the pre-coma and coma stage in cats, but we have no clue at present as to the nature of the acid or acids. Further studies should in-

[2] Since this was written, repeated determinations for acetone in the blood of comatose animals presenting indications of acidosis following adrenal extirpation, have shown that less than 3 mgm. per 100 cc. are present. Ketosis evidently is not responsible for the rise in organic acid.

clude besides serum non-protein nitrogen determinations to correct serum proteins,—hemoglobin (oxygen capacity) and cell volume measurements, in order to determine whether the blood concentration change is greater than is indicated by the change in serum protein values. The dog should be employed for experimental animal because of the large amount of blood needed for the various procedures.

Although data on these points are lacking in our own work, the investigations of several other workers indicate the results likely to be obtained. For examples, the serum non-protein nitrogen values (Swingle, 1926) are considerably higher in the completely epinephrectomized animals than in the unilaterally operated (normal) cats. Stewart and Rogoff (1925) have published some observations of the blood changes in epinephrectomized dogs such as hemoglobin, erythrocyte and leucocyte counts, conductivity of blood and serum with calculation of the number of cubic centimeters of serum in 100 cc. blood and the specific gravity of blood and serum. Their results indicate blood concentration of the double operated animals especially on the last day preceding death. These authors do not place too much stress upon the terminal concentration of the blood.

Rowntree (1925) states that he has observed several cases of individuals suffering from severe Addison's disease and which presented a shock-like condition, which seemed to show "blood concentration, as evidenced by its decreased water content and increased difficulty in obtaining venous blood. It is interesting to note also that two of the cases of Addison's disease described by Rowntree as in a shock-like condition showed a moderate acidosis." The acidosis was suspected on clinical grounds, and determination of the carbon dioxide capacity of the plasma revealed 31 and 36 volumes per cent respectively.

Protocols: *Cat ZM.* Small, gray, male weight 2830 grams. Right adrenal removed February 4, 4:30 p.m.; bled from the heart, February 14. No urethane given; venous blood obtained. Cat struggled only slightly. Left adrenal removed February 17, 3:30 p.m. Weight immediately after operation 2830 grams. Excellent condition February 20 at 12 m.; eats, plays and fights. Probably has accessory gland. Re-operated on the right side February 21. Region explored for accessory gland. Removed nodule of cortical tissue from the right side. Weight, 2760 grams, after operation. 8 a.m., February 22, typical adrenal symptoms at 2:30 p.m.; cat in coma; rapid, long-drawn respirations (37 per minute); bled with difficulty, blood greatly concentrated; 15 cc. of arterial blood obtained and about 30 cc. of venous; marked air-hunger present at time of bleeding. Died 3 p.m., February 22. The accessory gland sectioned; histological examination shows cortical tissue.

Cat ZN. Black and white, male. Right gland removed February 9, at 10 a.m. Bled from heart February 25, at 9 p.m. Six hundred milligrams urethane per kilo. Cat did not struggle. Left adrenal gland removed February 27 at 12 m. Weight after operation, 2807 grams. February 28, 9 a.m., symptoms present; marked weakness and drowsiness, 10 p.m., February 29. 5:30 a.m., animal in coma, unconscious; loud breathing; respiration 40 per minute; dilated pupils. Bled from the

heart; blood very concentrated. Marked air hunger. Weight at death, 2660 grams. This animal fed 40 cc. of milk by tube at 9 a.m., February 28.

Cat ZO. Large, black, male. Right gland removed February 14 at 10 p.m. Left adrenal removed February 17, 8 p.m. Animal fed 50 cc. milk 3 times daily. February 20 at 10 a.m., very bad symptoms; unable to walk; marked air hunger. Bled from the heart; venous blood. Cat went into convulsions when the blood was being withdrawn. First blood of this animal was not obtained.

Cat ZX. Gray, male. Left adrenal removed February 15, at 10 a.m.; bled from the heart at 9 a.m. No urethane given; venous blood obtained. Cat did not struggle. Right adrenal removed February 27 at 12:30 p.m. Weight, 2758 grams. February 28, 9 a.m., animal shows slight symptoms. 10 p.m., animal weak, but not in coma, or did not show marked air hunger. Symptoms slight. Bled from heart at 10 p.m. Animal did not struggle. Arterial blood obtained. Weight before bleeding, 2710 grams. Animal received 32 grams of milk, 8 p.m.

Note: At the time of the second bleeding the symptoms were not marked.

Cat SA. Small, white and black, male. Right gland removed February 27, 4 p.m. Bled from heart, March 4 at 12:30 p.m.; venous blood obtained. Cat did not struggle. No urethane given. Left adrenal removed March 7, 11:50 p.m. Weight, just before operation, 2135 grams. March 9 at 3 p.m., animal showed marked weakness. Eye sign present. Symptoms not marked; breathing fairly normal (27 per minute); weight 2055 grams. Bled at 3:30 p.m., venous blood obtained. No urethane given. Cat did not struggle. Survived bleeding 8 to 10 hours, dying between 9 p.m. and midnight.

Cat SC. Maltese, male. Right gland removed March 6; bled from heart, March 9 at 11 a.m. Venous blood. Cat did not struggle. March 16 at 3:30 p.m. re-operated. Left adrenal removed; weight 2310 grams. March 17 at 9:30 p.m., weight 2185 grams. Cat shows adrenal symptoms. 9:30 p.m., marked weakness, unsteady on feet, appears dehydrated. 12:30 a.m., cat very weak, but can walk; not in coma; breathes fairly normally; bled from heart. Venous blood. Animal died in convulsions at 1 a.m.

Cat SB. Maltese. Left adrenal gland removed February 28; bled from heart under urethane; 600 mgm. per kilo at 1:45 p.m. March 4. Cat did not struggle. Arterial blood obtained. Right adrenal removed March 8, at 11:30 a.m., weight 2688 grams. This animal was in very bad condition, March 8, 4 p.m. Second blood not obtained.

Cat V. Female. Operated December 18, 11 a.m. Right adrenal removed. Left adrenal removed December 19 under urethane; bled (800 mgm. urethane per kilo). 10 a.m., December 20, animal dying; typical adrenal death. Very fast respiration. Animal had to be given artificial respiration while bleeding. Note: Animal was dying and respiration ceased before the blood could be withdrawn; hence artificial respiration.

Cat ZE. Large, black and white, male. Weight 3240 grams. Right adrenal removed January 23 at 11:30 a.m. January 25, cat perfectly normal. Given 1.6 grams of urethane by stomach tube at 10 a.m. Bled from heart. Arterial blood obtained (10 cc. venous). This animal died of pneumonia, January 30. The second adrenal was not removed, nor was the blood test completed.

Cat ZF. Large, gray, male. Right adrenal removed January 23 at 12:30 p.m. Weight, 3032 grams. Bled from heart under urethane, January 25 at 12 o'clock. Venous blood obtained. Animal bled again January 29, without urethane. Cat did not struggle. Arterial blood obtained. Cat developed very sore mouth February 8. Salivates continuously. Refuses food. Fed daily by stomach tube. Left

adrenal removed February 8 at 12 o'clock. Animal under ether 38 minutes. Weight, 2951 grams. This animal developed typical adrenal insufficiency symptoms and died at 8:30 p.m., February 8 (e.g., lived 8½ hours). Weight 2931 grams. Lost 20 grams within 8 hours. *No blood obtained after second operation.*

Cat ZI. Maltese, male, adult. Weight 3000 grams. Right adrenal removed January 31 at 3:30 p.m. February 5, animal normal. Was given urethane (800 mgm. per kilo). Bled from heart at 12:15 p.m. Venous blood obtained. Cat did not struggle but began breathing rapidly after withdrawal of 25 cc. of blood. Animal normal at 8 p.m., February 5. February 8, left adrenal removed at 4 p.m. Weight of cat, 2940 grams. Under ether 32 minutes. February 9, animal salivating badly. Shows weakness in hind legs. Shows typical adrenal eye sign peculiar to cats. Refused food at 9 a.m. At noon, February 9, weight, 2860 grams. Skin dry and wrinkled, animal appears dehydrated. Had lost 80 grams within fourteen or fifteen hours. At 3 p.m., February 9, animal given 50 cc. milk by tube. Weight previous to feeding, 2851 grams,—a loss of 9 grams within three hours at room temperature of 73°. Animal did not urinate during this 3-hour period. At 5 p.m. the animal was bled from heart. Arterial blood obtained. Animal perfectly quiet while being bled. At time of bleeding cat was weak, drowsy, and if forced to move about, staggered uncertainly. Respirations were 37 per minute when bled. (This is fairly rapid, the average being 22 to 26 respirations per minute for cats.) Marked evidences of air hunger *were absent.*

Cat ZK. White and gray, male. Weight 2500 grams. Right adrenal removed February 2, 4 p.m. Bled from heart while under urethane (800 mgm. per kilo), 3:30 p.m. Arterial blood obtained. Animal did not struggle.

Cat died February 9 of pneumonia. Left adrenal not removed nor second blood taken.

SUMMARY

1. Heart-blood obtained from cats which had recovered from unilateral adrenalectomy was compared with that obtained after removal of the second adrenal gland. The blood serum was analyzed for the various acid ions, pH, and total base.

2. Consistent differences following bilateral adrenalectomy were:

a. A fall in pH, carbon-dioxide tension, serum bicarbonate, and total acid; indications of an uncompensated non-volatile acidosis.

b. This seemed to be due to an increase of phosphoric and organic acids.

c. Protein also rose, suggesting inspissation of the serum.

d. The nature of the changes is discussed.

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SEX-REVERSAL IN PARABIOTIC TWINS OF THE AMERICAN WOOD-FROG.[1]

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On the basis of his work on the free-martin F. R. Lillie (1917) expressed the view, "that sex-determination in mammals is not irreversible predestination, and that with known methods and principles of physiology we can investigate the possible range of reversibility." Numerous investigators followed this suggestion in an effort to determine the nature of the sex-differentiating factors.

Parabiosis experiments which were first performed in rats by Morpurgo in 1908, were carried out by Matsuyama (1919), Yatsu (1921) and Pfeiffer and Zacherl (1926). These authors obtained almost identical results in so far as there was no sex-change observed. Parabiosis here does not even prevent copulation or suppress fertility in either sex. Yatsu describes some pathological changes taking place in the ovaries and the uteri of the female, while the male co-twin is never impaired in the least by the female. But as the castrated male exerts the same influence as the normal one, it seems improbable, that we have to do in this case with a specific action of male differentiating factors.

The experiments were then extended upon birds and amphibians, in which the embryos are accessible for the experiment long before their sexual differentiation. Minoura (1921) first reported some intersexual features in the female chick embryos, caused by implanted pieces of the testes of adult cocks. But Greenwood (1925), when repeating that experiment did not find such influences. Recently Willier (1925) grafted embryonic

[1] The experiments were carried out in the Osborn Zoölogical Laboratory, Yale University. The microscopical study of the material was continued at the Marine Biological Laboratory, Woods Hole, Mass. The writer takes this opportunity of expressing his obligation to these institutions and feels also very much indebted to the International Education Board for the grant of a fellowship.

chick gonads, shortly before or after their sexual differentiation, into the chorio-allantoic membrane of nine-day-old host embryos. The material was preserved nine days after the operation. In no case of heterosexual combinations was there found a specific modification in the process of sex-differentiation of the graft, nor any trace of sex-reversal in the gonads of the host embryo.

Briefly, we can say that the experiments with mammals and birds failed to show a definite indication of a tendency towards a physiological sex-reversal.

On the other hand Burns (1925) reported a very successful experiment on the urodele *Amblystoma*. He joined young embryos during the tail-bud stage in parabiosis. Instead of the expected chance combinations of the sexes, requiring a ratio of 1 ♂♂ : 1 ♂♀ : 1 ♀♂ : 1 ♀♀, he obtained exclusively one-sexed pairs.[1] Burns is inclined to interpret the numerical result of 44 ♂♂ + 36 ♀♀ as a 1 : 1 ratio and infers, that in about one half of the original ♂♀ and ♀♂ combinations the males changed into females, while in the other half, the females changed into males. He claims, that "there is no prepotency, which constantly favors a given sex." But he did not happen to see any developmental stage of the supposed sex-transformation. He finds the pairs to be one-sexed already in the very earliest phases of sex-differentiation. Therefore the essential part of the process remains obscure. Burns' experiments, although successful in showing that a transformation of sex occurs, did not solve the problem of how it occurs.

In the spring of 1926 the writer started new experiments in which he joined young frog embryos in parabiosis. The operation was performed shortly after the closure of the medullary tube; that is, at an age of approximately 50 to 70 hours, when the eggs were kept in the laboratory at room temperature (18°–20° C.). In normal controls the first sex-differentiation takes place during the third week. In the parabiotic twins it is often somewhat delayed. The twins were preserved at intervals during the larval period and the stage of metamorphosis (Fig. 1).

The material belonged to four different species, but in the following we consider only the experiments with *Rana sylvatica*, the American wood-frog.

[1] ♂♀ means male on the left, female of the right side; and vice versa.

Controls were reared under identical laboratory conditions. Sex-differentiation was in full course by the 22d day, in a lot of larvæ, preserved at this time. The animals metamorphosed between the 44th and the 59th day. The species proved to

FIG. 1. Parabiotic twins TS1. Newly metamorphosed. The left side animal with the pigmented ventral surface is an European *Temporaria*, the right side animal is an American *Sylvatica*.

belong to the differentiated sex type. Among these 196 controls were 100 females and 96 males—a very close approximation to a 1 : 1 ratio (expectation calling for 98 : 98 with a probable error of $\pm$ 4.7).

We should therefore expect, that by chance combination, the 56 twins considered in this paper would consist of the following zygotic groups—14 ♂♂ + 14 ♂♀ + 14 ♀♂ + 14 ♀♀ (the probable error being $\pm$ 2.2). Preliminary observations on a part of this material seemed to indicate that in the heterogeneous combinations the sex of one animal is not influenced by that of the other, and a statement to this effect was made in an evening lecture at the Woods Hole Laboratory this summer.

There are indeed a considerable number of cases like No. 44, in which one animal possesses normal testes, while the other has ovaries, which do not show any apparent deviation from the

FIG. 2. Parabiotic twins SS44. (Both are *Rana sylvatica*.) Left larva, 34 mm. total length. 68 days after the operation. Male. Transverse section through one of the testes. The germ cells (primary spermatogonia) are in the sex cords. × 444.

typical female conditions (compare Figs. 2 and 3). Later investigations, however, showed, that some of the females twinned with males underwent sex-reversal. In three such cases large parts of the gonads show a nearly typical testicular structure (Fig. 4), while other parts are ovarial or show progressive stages in the transformation process. In seven more cases the gonads are in the first stages of that transformation (Fig. 5). Such ovaries are, without exception, very slender. The same thing is observed in some cases, in which the reorganization has not yet begun. Obviously an *inhibition in ovarial growth* is the first visible effect exerted by the male co-twin.

From the fact, that the reverse combination of a female with a sex-changing co-twin was never found, it becomes evident, that *the male sex dominates the female one.*

FIG. 3. Same pair SS44. Right larva, same length as left one. Female. Transverse section through one of the ovaries. The germ cells (primary oögonia and auxocytes) are in the peripheral germinal epithelium. *og.*, primary oögonia. × 444.

With regard to the last two points, the above statements are in full agreement with Lillie's observations on free-martins. However, while the latter never effect a complete sex-change, and do not produce male germ cells, the changing frog twins apparently are on the way to become true males. In frogs, ex-

FIG. 4. Parabiotic twins SS40 (*R. sylvatica*). Right animal shortly after metamorphosis. 68 days after the operation. Female changing into male. Transverse section through the posterior part of the gonad which shows nearly typical male conditions. Highly developed sex cords, relatively small number of spermatogonia. Rests of the former germinal epithelium at *mig.* (migrating germ cells) and *deg.* (degenerating oögonia). × 444.

hibiting a transformation of sex, the undifferentiated germ-cells, which in the ovary are exclusively found in the peripheral germinal epithelium (so-called primary oögonia, Fig. 3, *og.*) migrate to the center of the gonad (Fig. 5, *mig.*). They join the sex-cords, to form the characteristic lobules, which are known to be the anlagen of the seminiferous tubules (Fig. 4), (Witschi, 1914; Swingle, 1926). From this stage they are therefore to be considered as *spermatogonia* (Fig. 4, *spg.*).

The described facts are in many respect in apparent contrast

to those reported by Burns (1925) in *Amblystoma*. This author did not find that the male sex predominates or even that sex-transformation decreases the growth of the involved gonads. Furthermore, he supposes that the change of sex takes place before the time of sex-differentiation. Let us consider the latter point with respect of the present experiments with *R. sylvatica*.

FIG. 5. Parabiotic twins SS41 (*R. sylvatica*). Left larva, 38 mm. total length. 68 days after operation. Female changing into male. Transverse section through one of the gonads, showing inhibition of growth, disintegration of the germinal epithelium and formation of the testis in the center of the gonad. *mig.*, germ cells migrating into the sex cords. × 444.

Supposing there were such an early sex-reversal in the frog twins, then the number of one-sexed pairs ought to be increased at the expense of the two-sexed ones. However, the statistical evidence is against such an assumption. There were found the following combinations:

♂♂—16
♂♀—17 (seven of the females undergoing sex-reversal)
♀♂—10 (four of the females undergoing sex-reversal)
♀♀—13

The result of 29 one-sexed and 27 two-sexed pairs is quite close to expectation (28 ± 2.5). Among the total of 112 individuals we find 59♂ and 53♀ (the latter group including the sex-changing animals). The deviation from the mean is less than the single P.E. It can be considered, therefore, as well established, that *there was no sex transformation before the time of sex-differentiation.*

In discussing the fact of the dominance of the male sex in the free-martin, Lillie is inclined to consider the time-factor as an essential one. The observations of his students (Chapin, 1917 and Bascom, 1923), relating to the earlier development of interstitial cells in the testes and the later differentiation of the ovaries, seem to favor the theory of dominance of the male twin by earlier hormone production. Lillie admits, however, that other causes may also exist. In the case of our frog twins it is evident that the time-factor has not the suggested significance. The male-differentiating factor reaches the female gonads after the ovaries have acquired a relatively advanced stage of development. This means, that the female-differentiating factor is already in full action. In the resulting competition of both, the male-differentiator acts first as an inhibitor and later on as a suppressor of the female one. This is in strict agreement with the conclusions derived from the author's observations on hermaphroditism (1914; 1921). I think, that there can no longer be any doubt about *the antagonistic character* of these two factors.

Lillie (1923) and Bissonnette (1924) report on some earlier stages of the free-martins with rather highly developed ovarial structures. It seems not improbable that the female co-twins started with a typical female sex-differentiation. Such cases would lead to the same conclusions as the parabiotic frog twins. The assumption, that only the male is producing hormones in an early time, would apparently not be sufficient to explain the suppression of the active ovarial development. There seems to be no escape from the postulated sexual antagonism.

Some years ago Goldschmidt (1920) put forward the idea, that the sex-determining genes act like enzymes, stimulating the production of sex differentiating hormones. The term

"hormones" taken in its proper sense, would indicate, that the sex-differentiators circulate in the blood and are spread throughout the entire body. The writer's previous studies on sex-differentiation in the frog seem to point in a somewhat different direction. In hermaphrodites the eggs are formed exclusively in the germinal epithelium, while the spermatogonia develop in the sex-cords. At the beginning of the sex-reversal from female to male those germ-cells situated closest to the sex-cords are the first to show characteristic changes and to migrate into the sex-cords. If we consider the behavior of the germ-cells as an indication of, or a reaction to, the nature of their environment, then we must attribute male differentiating properties to the sex-cords. On the other hand, from the fact that the germ-cells which were not attracted by the sex-cords, but which remained in the germinal epithelium, become eggs, it becomes evident that this epithelium includes female-differentiating properties. From such facts, which furthermore are supported by experimental data, I proposed the *theory of localized sex-differentiators* ("lokalisierte Innenfaktoren," 1914). These probably are comparable to Spemann's "Organisatoren."

The fact of independent sex-differentiation in genetically two-sexed twins favors again that localization theory. Regarding the secondary sex-reversal of the female part, the coöperation of hormones is not improbable. We may assume, either that part of the active substance elaborated in the sex-cords is carried away by the blood-stream, or that the differentiated testes produce a hormone, which is different from the substance inducing the first sex-differentiation. Yet, it is to be kept in mind, that—at least in the frogs—there is no definite proof, that the testes of the male twin act by way of the blood-stream. This question has to be discussed in connection with a detailed study of the homoplastic and heteroplastic twins, which will be published at a later date.

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THE PREVENTION OF TETANY BY ORAL ADMINISTRATION OF MAGNESIUM LACTATE

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Stimulated by the investigations of MacCallum and Voegtlin (1909) on the relation of parathyroid tetany to calcium metabolism, Berkeley and Beebe (1909) made a detailed study of the effect of salts other than calcium upon the tetany syndrome. They were able to demonstrate that intravenous injections of strontium and magnesium following bleeding were effective in causing the disappearance of the violent neuro-muscular symptoms, and furthermore, that coincident with the alleviation of symptoms there occurs a marked decrease in electrical excitability of the peripheral nerves. On the basis of their work they stated that since strontium and magnesium, when injected, temporarily relieved tetany, and since both salts appeared to be as efficacious as calcium, the latter could hardly be said to be specific in the treatment of tetany. Magnesium, they stated, is a powerful depressant and is dangerous to life when given in a dose sufficient to control the symptoms of tetany.

In 1911 Voegtlin and MacCallum, working on the influence of various salts upon tetany, found that the symptoms of tetany can be temporarily relieved by intravenous injections of magnesium, thus confirming Berkeley and Beebe's work. They showed that magnesium causes a decrease in the electrical excitability of the motor nerves and brings about a general state of anesthesia in both normal dogs and dogs in tetany. Although all tetany symptoms disappeared following intravenous injections of magnesium salts, Voegtlin and MacCallum were unable to keep the dogs alive more than seven days—the animals invariably succumbing to tetany.

Previous work by the writer (Swingle and Wenner, 1926) had demonstrated that by continuous oral administration of strontium, parathyroidectomized dogs can be kept free from tetany for long periods and if treated sufficiently long may eventually permanently recover.

The present work was undertaken with the object of determining whether or not magnesium salts are likewise efficacious in preventing tetany and if so what is the mechanism of their action in relation to blood calcium and tetany prevention. Survey of the literature, and previous work (Swingle and Wenner, 1926) on the effect of bleeding upon the alleviation of tetany,

had convinced the writer that if magnesium salts are effective in the treatment of tetany, they evidently exert some influence upon calcium metabolism. It was considered unlikely that magnesium *per se* could substitute for calcium. With this object in view the following experiment was undertaken.

The writer takes this opportunity of acknowledging his gratitude to Prof. W. W. Swingle for suggesting the problem, and for his untiring interest and kindly criticism throughout the progress of the work.

Ten dogs were used in this investigation. One grain of morphine sulphate and 1/150 grain atropine sulphate dissolved in 2 cc. of sterile water were injected one hour or more before the operation. This procedure is very beneficial for morphine causes the dog to take ether well and atropine prevents salivation. All parathyroids were identified before the thyroids and surrounding fascia were removed. After excision, the thyroids and connective tissue were again examined and the parathyroids again identified. It is quite evident that by this method all parathyroids were removed. All dogs developed tetany some time after operation; those dogs that did not show tetany symptoms within the usual period were fed large quantities of meat, and tetany followed within eighteen hours.

The animals were kept on a modification of the diet used by Cowgill (1923) for metabolism experiments. The diet mixture consisted of casein 38.8 per cent, sucrose 35.3 per cent, lard 17.6 per cent, butter 7.8 per cent, and salt mixture (sodium chloride 10 grams, ferric citrate 4 grams and few drops of Lugol's solution) 0.6 per cent (Swingle and Rhinhold, 1925). Approximately 16 grams of this mixture per kilo body weight were fed the animals daily. Following the onset of tetany, when the dogs refused food, warm water was added and the diet mixture administered by stomach tube. The use of this diet does not delay in any way the onset of tetany in dogs. This fact has been adequately demonstrated by several investigators (Inouye, 1924) (Swingle and Wenner, 1926).

Magnesium lactate in 5 per cent solution was given by stomach tube three times daily in 100 cc. doses. This amount was decreased at times for occasionally one of the dogs would become stuporous due to the anesthetic effect of the magnesium. The dosage was lessened also at later periods when the experiment was drawing to a close. The medication began in all cases except dog 10, a few hours after operation, when the animals had recovered from the effects of the anesthetics.

Milk was added to the diet of those dogs that showed marked tetany symptoms for several consecutive days. Magnesium lactate given at this time did not bring about an increase in the calcium content of the serum, but, through its depressing action on the central nervous system, concealed the more violent symptoms of tetany. The rise in calcium was obtained

by giving one-half pint of milk along with magnesium. Although milk contains only 1.2 grams of calcium per liter, one-half pint given daily for a few days is sufficient to bring the serum calcium above the tetany level when administered along with magnesium. When this new calcium level was attained milk was discontinued. Further administration of magnesium lactate tended to keep the serum calcium at this point. Dog 3 remained in tetany from the third to the tenth day. The animal received one-half pint of milk daily from the eighth to the eleventh day. Tetany disappeared on the tenth day and on the twelfth day, when the milk was discontinued, the serum calcium was 8.1 mgm. per 100 cc. Dogs 3, 7, 8, 9, 10 received milk whereas milk was not given to the remaining five. The data of two of the ten experimental dogs are summarized in table 1.

Serum calcium was determined at the time of operation and at varying intervals thereafter. Clark and Collip's (1925) modification of Kramer and Tisdall's (1921) method for serum calcium determination was used. Blood was aspirated directly from the left ventricle of the heart. This method of drawing blood was employed because of its simplicity. The withdrawal of large quantities of blood is to be avoided for following hemorrhage the serum calcium level is elevated (Swingle and Wenner, 1926) and the pH falls (Bennett, 1926). Since 20 cc. of dogs' blood will produce only 6 to 7 cc. of serum, calcium determinations were not made as often as might have been desired. Figures 1 and 2 show the plotted curves of the serum calcium of dogs 2 and 3.

Dog 2, figure 1, never showed symptoms of tetany up to the eighteenth day following operation. Serum calcium remained at about 8 mgm. per 100 cc. for twelve days. On the eighteenth day the dog was fed a large quantity of meat. Violent tetany occurred on the following day, serum calcium then was 6.1 mgm. Three hundred cubic centimeters of a 5 per cent solution of magnesium lactate were given. Five hours later tetany disappeared. The calcium on the twentieth day was 8.97 mgm. Tetany never again appeared. Magnesium therapy was discontinued on the twenty-eighth day, when the dog was placed on a meat diet without ill effects resulting. It is interesting to note that this dog received no milk.

From the results obtained with strontium lactate and magnesium lactate it was considered probable that the lactates of other cations would be as effective if the dosage could be carefully regulated. With this idea in mind, sodium and potassium lactate were tried. Cadmium chloride was also used. However, cadmium chloride and cadmium and sodium lactate proved to be so irritating to the gastro-intestinal tract that 100 cc. of $\frac{1}{2}$ of 1 per cent solution will cause the most violent vomiting. Potassium lactate produced no gastric disturbance but was practically inert as a therapeutic agent in the treatment of parathyroid tetany. Potassium lactate was administered to three parathyroidectomized dogs. Tetany

appeared, in all cases, in the usual time followed by death several days later. Potassium lactate in large amounts, 300 cc. of a 5 per cent solution, had no effect upon relieving tetany. These findings with potassium lactate agree with those obtained by Voegtlin and MacCallum (1911). Table 2

TABLE 1

DATE	TIME	SERUM CALCIUM	MAGNESIUM LACTATE GIVEN	REMARKS
Dog 7. Terrier ♂, 7 kilos				
1926		*mgm. per 100 cc.*		
Jan. 13	4:00 p.m.	10.7	300 cc. 5 per cent solution	Thyroparathyroidectomy
Jan. 14–17				Normal
Jan. 18	11:30 p.m.	5.9	300 cc. 5 per cent solution	Tetany
Jan. 19–20			300 cc. 5 per cent solution	No tetany
Jan. 21			300 cc. 5 per cent solution	Slight tremors chattering of teeth
Jan. 22–28			300 cc. 5 per cent solution	1 pint of milk given daily
Jan. 29		10.0	100 cc. 5 per cent solution	Milk discontinued
Feb. 4			100 cc. 5 per cent solution	Normal
Feb. 5		7.5	100 cc. 5 per cent solution	No tetany
Feb. 6–8			100 cc. 5 per cent solution	No tetany
Feb. 9	4:00 p.m.	6.2		Slight tetany. Muscular twitching. No magnesium lactate
Feb. 10	3:00 p.m.		5 gm. dissolved in ½ pt. of milk	Tetany
	9:00 p.m.		5 gm. dissolved in ½ pt. of milk	Slight improvement
Feb. 11			300 cc. 5 per cent solution given	Recovered, ½ pint milk
Feb. 12–17			100 cc. 5 per cent solution	Normal. No milk given
Feb. 18		7.6	100 cc. 5 per cent solution	Normal. No milk given
Feb. 19–24			100 cc. 5 per cent solution	No tetany
Feb. 25–26		7.6	50 cc. 5 per cent solution	No tetany

TABLE 1—Continued

DATE	TIME	SERUM CALCIUM	MAGNESIUM LACTATE GIVEN	REMARKS
Dog 7. Terrier ♂, 7 kilos—*Continued*				
1926		*mgm. per 100 cc.*		
Feb. 27	8:30 a.m.		50 cc. 5 per cent solution	Tetany, ½ pint milk given with magnesium lactate
	2:30 p.m.			Recovered. Animal apparently normal
Feb. 28–Mar. 6			100 cc. 5 per cent solution	Normal
Mar. 7–8				No magnesium lactate given. Dog fed small portions of meat
Mar. 9		9.3		Normal
Mar. 10–18				Dog normal
Mar. 19	3:00 p.m.		300 cc. 5 per cent solution	Violent tetany. Enema given
	6:00 p.m.			Condition improved; ½ pint milk given
Mar. 20			300 cc. 5 per cent solution	Tetany continues. Twitching not so marked, 1 pint milk given
Mar. 21–22			200 cc. 5 per cent solution	Dog better. 1 pint milk given
Mar. 23–24			100 cc. 5 per cent solution	1 pint milk daily
Mar. 25–26				Dog normal. Fed 1 pint milk bread and small piece of meat
Mar. 27		10.1		Normal. Placed on meat diet. No magnesium lactate given
Mar. 28–Apr. 21				Normal
Apr. 22	11:30 a.m.			Tetany. Animal had not defecated for about 3 days. Enema given. No magnesium lactate administered
	4:30 p.m.			Tetany symptoms gone
Apr. 23–27				Normal
Apr. 28	3:45 p.m.	9.5		Normal (splenectomy)
June 10				Chloroformed

TABLE 1—*Concluded*

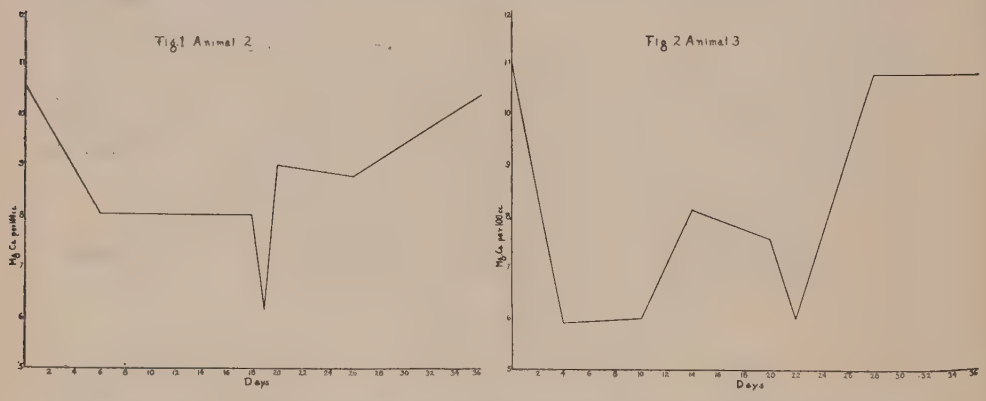
DATE	TIME	SERUM CALCIUM	MAGNESIUM LACTATE GIVEN	REMARKS
Dog 9. Mongrel ♂, 20 kilos				
1926		*mgm. per 100 cc.*		
Feb. 1		11.0		Thyroparathyroidectomy
Feb. 2–9			300 cc. 5 per cent solution	Normal
Feb. 10	10:30 a.m.		100 cc. 5 per cent solution	Tetany
	3:00 p.m.	6.5	100 cc. 5 per cent solution	Tetany very severe
	9:20 p.m.	6.3	100 cc. 5 per cent solution	Animal greatly improved. ½ pint milk given
Feb. 11–12			300 cc. 5 per cent solution	Normal, ½ pint milk given
Feb. 13–17			300 cc. 5 per cent solution	Animal is very inactive. No milk
Feb. 18–19			300 cc. 5 per cent solution	Very stuporous. Given 1 pint milk
Feb. 20–22			300 cc. 5 per cent solution	Slight muscle tremors
Feb. 23–24			300 cc. 5 per cent solution	No tetany. Animal stuporous
Feb. 25–Mar. 5			200 cc. 5 per cent solution	Dog normally active
Mar. 6		10.1		Magnesium therapy discontinued. Dog placed on meat diet
Mar. 7–24				Normal
Mar. 25		10.3		Normal
Mar. 26–Apr. 10				Slight tetany. Dog has not defecated for 2 days. Enema given. Tetany disappeared 3 hours later
Apr. 11–May 9				Normal
May 10		10.8		Normal
May 13				Normal. Used for other experiments

shows the ineffectiveness of cadmium, sodium and potassium in the treatment of tetany.

Six parathyroidectomized dogs were treated with cadmium lactate, one with cadmium chloride and two with sodium lactate. Both cadmium and sodium salts produced violent vomiting which hastened the appearance of

tetany symptoms. Vomiting causes a loss in acid and if long continued results in tetany possibly due to alkalosis. In this case the withdrawal of acid combined with the absence of the parathyroid hormone was responsible for the early onset of tetany. Typical tetany spasms occurred within thirty-six hours after operation and death followed, on the average, within three days. It was thought at the time that sodium lactate, if assimilated, would cause an increase in the sodium ion and bring on tetany much sooner. Although tetany did appear at a much earlier period it was not due to an increase in the sodium of the blood caused by the assimilation of sodium lactate for all of the sodium lactate given by stomach tube was vomited immediately.

The fatal results obtained by the use of cadmium, potassium and sodium serve very convincingly as proof that the results obtained by the use of



Figs. 1 and 2. Curves showing the effect of magnesium on the level of the serum calcium. Dog 3 received milk when the low points on the curve were reached Dog 2 received no milk.

magnesium could not be due to any failure in removing all parathyroid tissue at the time of operation since the writer performed all operations and used the same technique.

From the data presented here it would appear that magnesium in some way aids to some extent, other than through its depressant action, in rendering the calcium more soluble thus raising the content of this element in the blood. Greenwald (1925) has suggested that in the absence of the parathyroid hormone, calcium phosphate becomes less soluble and is precipitated in the tissues and in this way is removed from the blood stream rather than by elimination from the organism through the gut wall as proposed by Salvesen (1923). It may well be that a bivalent cation other than calcium, when in excess may unite with the phosphate and thus liberate the calcium in a more soluble form and place it in the

TABLE 2

The effect of lactates of cadmium, sodium and potassium

ANIMAL	DATE OF OPERATION	DATE TETANY FIRST APPEARED	LIFE SPAN	AMOUNT SALT GIVEN DAILY	REMARKS
				Cadmium lactate	
	1926		*days*		
1	Mar. 16	Mar. 17	3	100 cc. 2 per cent solution cadmium chloride	Salt caused violent vomiting and frothing at mouth
2	July 7	July 9	3	100 cc. 5 per cent solution cadmium lactate	Violent gastric disturbance caused by cadmium lactate. Animal died of typical tetany symptoms
3	July 7	July 10	4	100 cc. 1 per cent solution cadmium lactate	Violent gastric disturbance caused by cadmium lactate. Animal died of typical tetany symptoms
4	July 8	July 9	2	100 cc. 1 per cent solution cadmium lactate	Died in convulsions. Vomited entire amount of cadmium solution
5	July 10	July 12	2	100 cc. 0.5 per cent solution cadmium lactate	Died in convulsions. Vomited entire amount of cadmium solution
6	July 15	July 17	4	100 cc. 0.5 per cent solution cadmium lactate	Died in convulsions. Vomited entire amount of cadmium solution
7	July 15	July 17	2	100 cc. 0.5 per cent solution cadmium lactate	Died in convulsions. Vomited entire amount of cadmium solution
				Sodium and potassium lactate	
1	July 20	July 22	3	200 cc. 1 per cent solution sodium lactate	Dog vomited after every administration of sodium lactate. Died in convulsions
2	July 20	July 21	1	300 cc. 0.5 per cent solution sodium lactate	Dog vomited after receiving solution. Died in tetany
3	Oct. 4	Oct. 7	8	300 cc. 5 per cent solution potassium lactate	Solution retained. Tetany never abated once it appeared
4	Oct. 4	Oct. 8	4	300 cc. 5 per cent solution potassium lactate	Tetany appeared on the 4th day. Potassium lactate had no effect in relieving tetany
5	Oct. 5	Oct. 9	14	300 cc. 5 per cent solution potassium lactate	Tetany appeared on 4th day and continued until death occurred

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blood. However, once animals develop tetany magnesium administration exerts little effect other than to disguise the more violent symptoms through its anesthetic action. The serum calcium when below the tetany level, can be raised in most cases only by administration of calcium in some available form. Milk is a very good source of calcium and for this reason was chosen for the purpose of increasing the serum calcium. When this rise in serum calcium is secured, continued magnesium therapy tends to maintain the calcium content of the serum at this new point.

Table 1 and figures 1 and 2 show that on the average, operated dogs receiving magnesium develop tetany at later periods than untreated dogs. It is well known that dogs receiving no medication show tetany symptoms within three or four days. Dog 10 received no magnesium following operation, developed tetany within twenty four hours, whereas all other

TABLE 3

Effect of magnesium in delaying onset of tetany

ANIMAL	DATE OPERATED	Ca NORMAL	DATE TETANY	Ca	INTERVAL OPERATION—TETANY
		mgm.		*mgm.*	*days*
1	Oct. 19	10.6	Oct. 24	5.8	5
2	Oct. 23	10.3	Nov. 11	6.1	19
3	Oct. 29	10.9	Nov. 2	5.8	4
4	Nov. 4	10.5	Nov. 7	7.1	3
5	Jan. 13	10.7	Jan. 18	5.9	5
6	Jan. 13	11.5			
7	Jan. 13	10.7	Jan. 18	5.9	5
8	Feb. 1	11.6	Feb. 15	5.7	14
9	Feb. 1	11.0	Feb. 10	6.5	9

dogs who were given magnesium immediately after operation showed symptoms of tetany on the fourth to twentieth day. Animal number 6 remained normal for ten days after operation but escaped from the laboratory before the completion of the experiment.

It has been demonstrated by Salvesen (1923) that milk contains enough calcium so that if fed in large quantities to parathyroidectomized dogs it will maintain the animals in a normal condition for long periods. Swingle and Wenner (1926) in experiments on the effects of strontium on parathyroidectomized dogs, have shown that dogs which develop tetany may be brought back to normal within a few days, by adding one pint of milk to the amount of strontium given per day. With magnesium administration alone it is difficult to bring about a recovery once tetany becomes manifest. The animals may be kept alive for some time by continuous magnesium treatment but the blood calcium remains below the tetany level, except

in the case of dog 2 where the disappearance of tetany following the administration of 300 cc. of a 5 per cent solution of magnesium lactate was accompanied by a rise in serum calcium from 6.1 mgm. to 8.97 mgm. per 100 cc. It was found that one-half pint of milk given along with magnesium daily for a few days will cause the abatement of tetany which is coincident with a rise in calcium level of the serum.

By this careful administration of magnesium for about forty days, parathyroidectomized dogs may permanently recover from tetany and, at the end of this time, may be placed on a full meat diet and the magnesium treatment discontinued. Thus dogs 2, 3, 7, 8 and 9 completely recovered and became adjusted to the loss of their parathyroids after periods of medication lasting 27, 28, 73, 56 and 34 days respectively. Animals 7 and 8 had many attacks of tetany during the periods of medication; in these cases the period was greatly lengthened.

Several interpretations have been ascribed to the readjustment that takes place in the body following the loss of the parathyroids. It has been suggested by Luckhardt and Rosenbloom (1922), Swingle and Wenner (1926) and others, that the recovery may be due to hypertrophy of accessory parathyroid tissue, or possibly to a compensatory action of other organs. However, this readjustment, as Inouye (1924) pointed out, is not a definitely fixed one and does not indicate the dispensability of the parathyroid glands. Two dogs reported by Inouye developed tetany after remaining free from tetany for long periods. Animals 7 and 9 reported here, developed tetany on the 27th and 36th day respectively after the magnesium administration had been discontinued and were on a full meat diet. It was noted that for some reason, probably lack of exercise, these dogs became constipated. Following the administration of enemata, tetany symptoms soon disappeared and never again recurred.

The importance of constipation in relation to tetany has been stressed by many investigators. Luckhardt and Rosenbloom (1922) observed that parathyroidectomized dogs may be kept alive indefinitely if given intravenous injections of Ringer's solution and if care is taken that the animals do not become constipated. Swingle and Wenner (1926) found that dogs kept free from tetany by strontium develop tetany very quickly if constipated. This has also been found true in experiments where calcium, lactose and galactose have been the therapeutic agents. It appears that the end products of intestinal putrefaction may exert an influence upon the predisposition of parathyroidectomized dogs to tetany, as Dragstedt maintained. All animals reported here were given enemata at various intervals during the experiment despite the fact that magnesium lactate acts to some extent as a laxative agent.

Magnesium acts in a way similar to calcium and strontium. As shown by Voegtlin and MacCallum (1911), the sedative effect on the nervous

system produced by magnesium is much greater than that produced by calcium or strontium. In dogs in tetany, it has been found here that oral administration of magnesium readily abolishes the extreme neuromuscular symptoms, and in some cases it renders the animal quite anesthetic. However, with the removal of the symptoms recovery does not follow the disappearance of tetany. An analysis of the blood reveals that the calcium is increased but to a small degree. If now calcium is supplied in amount just large enough to cause a rise in serum calcium, the magnesium tends to keep the serum calcium at this new level.

This fact would indicate that magnesium acts in another manner aside from its anesthetic action upon the nervous system. It is suggested that the magnesium, when in excess, takes care of the increased phosphates or in some way brings the calcium back into solution.

The result detailed here concerning the permanent recovery after forty days' treatment with magnesium, are not unique since there are several accounts in the literature of permanent recoveries brought about by treatment with various agents for a period of forty or more days. Luckhardt and Rosenbloom (1922) found that animals could be cured of all tetany symptoms following parathyroidectomy by continued intravenous injections of Ringer's solution. They state that Ringer's solution in which strontium had been substituted for calcium is almost as effective. After a period of four to five weeks of this treatment the dogs permanently recover and can be placed on a meat diet without ill effects. Dragstedt and Peacock (1923) kept dogs free from tetany indefinitely on a diet of lactose, bread and milk *ad libitum*. After six weeks their animals took a stock diet and remained normal for long periods. Luckhardt and Goldberg (1923) were able to keep dogs free from tetany by calcium therapy. Inouye (1924) found galactose as well as lactose efficacious in the prevention of tetany. Collip (1925) reported two dogs that were returned to the kennels as normal dogs after they had received daily injections of parathyroid extract for two or three weeks. Dragstedt (1925) and Swingle and Wenner (1926) found strontium an effective agent in the prevention of tetany. After forty days of strontium therapy dogs returned to normal, were placed on a full meat diet and the therapeutic agent discontinued.

The problem of why dogs permanently recover after a certain period of treatment with magnesium, is as yet unsolved. The same end is brought about by intravenous injections of Ringer's solution, administration of strontium, calcium and magnesium salts and diets containing large amounts of lactose and galactose, although the actions of the various substances administered are quite different. Whatever the actions of these substances may be, the ultimate readjustment that occurs in the organism after forty or so days of that specific treatment, must be the same.

It has been suggested that this adjustment is brought about by 1, the

hypertrophy of accessory parathyroid tissue, or 2, by other organs taking over the function of the parathyroid glands. Dragstedt, Phillips and Sudan (1923) observed that recently parathyroidectomized dogs are less resistant to toxins than dogs recovered for long periods. There is an increase in resistance or gradual return to equilibrium or normalcy. This gradual return has been noted here also where dogs at the end of the experiment required less magnesium than dogs recently operated upon. This gradual return could support either view. However, this final recovery does not indicate the dispensability of the parathyroid glands for, as pointed out before, any abnormal condition is capable of disturbing the equilibrium established in those "recovered" animals, with tetany resulting.

Recently in a series of experiments (Wenner, 1926) the writer has been able to keep parathyroidectomized dogs free from tetany by oral administration of ammonium chloride. After forty to sixty days of such treatment the ammonium chloride can be discontinued and the animals placed on a heavy meat diet without tetany resulting.

SUMMARY

1. Continuous oral administration of magnesium lactate is an effective agent in the prevention of tetany in parathyroidectomized animals.

2. Magnesium tends to keep the calcium content of the blood serum above the tetany level. It is suggested that magnesium, by uniting with the excess phosphorus, keeps the calcium in solution.

3. The sedative effect of magnesium greatly reduces the excitability of the central nervous system whereby the more violent symptoms of tetany are held in check.

4. Magnesium-treated thyroparathyroidectomized dogs, kept free from tetany for about forty days, become readjusted to the loss of the parathyroids and may permanently recover and may be placed on a full meat diet without ill effects.

5. The lactates of cadmium, sodium and potassium, tried in connection with the magnesium experiments, were found to be ineffective in preventing or relieving tetany.

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ADDISON EMERY VERRILL AND HIS CONTRIBUTIONS TO ZOOLOGY.

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Systematic Zoology loses one of its American pioneers in the death of Addison Emery Verrill, Professor of Zoology, emeritus, at Yale University, which occurred on December 10, 1926, during a visit to Santa Barbara, Calif. At the time of his death Professor Verrill was within two months of having completed his eighty-eighth year. He was associate editor of this Journal from 1869 to 1920, and contributed to it a large number of his briefer taxonomic papers. To Verrill, zoological progress is permanently indebted for having made known to science probably a larger number of new species of marine invertebrates than any other American worker. The exact number of such hitherto undescribed forms which he discovered was not known even to Verrill himself, but he often stated that it was well above a thousand. He never found time to summarize many of his earlier investigations, although he never forgot any of them. His constant urge was to move on into new fields when the outstanding products of the old were exhausted, postponing for a later time the less promising objects.

The type specimens of most of Verrill's species are among the zoological collections of the Peabody Museum of Natural History at Yale University. With only meager assistance, much time was consumed in the routine of sorting and labeling of the vast amount of material which came to the Museum through his connection with the United States Fish Commission. The single assistant connected with Verrill's department for many years, Dr. Katherine J. Bush, proved to be so able an investigator that she was assigned by him to independent research and became a recognized authority on certain groups of mollusks and annelids.

In 1864, when Louis Agassiz, at Harvard, and Joseph Leidy, at Philadelphia, and a few others had developed the study of animal life to such an extent that zoology had begun to be recognized in America as one of the sciences, Yale College called upon Agassiz for a young man who would bring the new science to New Haven. Fortunately Agassiz then had as assistant in the Museum of Comparative Zoology the man who served as professor of zoology at Yale for forty-three years, until he was retired in 1907.

When appointed at Yale, Verrill was 25 years of age, having been born at Greenwood, Me., February 9, 1839. Before his thirteenth year he had learned to recognize the varieties of rocks and minerals to be found in his native town and had assembled a collection of considerable size. The following year he moved with his parents to Norway, Me., and turned his attention to the study and collection of wild flowers. He soon had an herbarium of several hundred species, each of which he could recognize and name almost instantly throughout the rest of his life. At seventeen he made a collection of the local shells, insects, amphibia, reptiles, bird and mammals, making the identification, when possible, with the aid of such few books as were available and noting especially the kinds which were different from any described in his books. In this way, and almost wholly without other assistance, he laid a broad foundation for the taxonomic studies which were to constitute his life work.

In order that he might enter Harvard College and study under the eminent Louis Agassiz, young Verrill attended the local academy, known as the Norway Liberal Institute, and in May, 1859, arrived in Cambridge. He often spoke of his amazement in finding that Agassiz did not give regular courses to his students, but allowed each beginner to select a special problem for investigation. The topic chosen, however, could not be laid aside until it had been mastered in every detail. In his Junior year, 1860, he was appointed by Agassiz as assistant in the Museum of Comparative Zoology, and after receiving his degree of B.S. in 1862 continued two years longer in this position.

The versatility of his boyhood studies in natural history now began to bear fruit, for in the years 1862 and 1863 he published no less than twenty-two papers, of which two were on minerals, one on plants, three on corals and their allies, seven on birds, four on mammals, three on amphibians and the

others on general natural history. Most of these were brief taxonomic papers or lists of local species, but one of them, on the revision of the Polypi of the eastern coast of the United States, showed a remarkable comprehension of the principles of taxonomy and formulated the classification used at the present time.

On his arrival at Yale, Professor Verrill immediately set about the development of a zoological museum out of the old "Natural History Cabinet" in one of the small buildings of the University, but cramped quarters gave little opportunity for exhibits. The building of the Peabody Museum of Natural History offered space for more extensive exhibits and he, with Professor Sidney I. Smith and several assistants, prepared and arranged a zoological collection for public exhibition which for many years compared favorably with that of any college museum in the country. Professor Verrill remained in charge of the zoological collections until 1910, by which time they had increased until they contained more extensive series of marine invertebrates than were to be found in any American museum with the exception of the United States National Museum and the Museum of Comparative Zoology at Harvard.

The building which held these collections was removed in 1916 and the zoological material was later installed in the new Peabody Museum of Natural History, where it is now available for reference and further comparative studies.

It may be of interest to recall that Verrill lived through practically the entire history of zoology in America; from the coming of Louis Agassiz in 1847, to the experimental period of the present century. But while the vogue of the science changed from taxonomy to comparative anatomy, and then to adaptations and the evidences of evolution, later to biometry, regeneration, cell lineage and embryology and, at the beginning of the century, to experimental fields and genetics, Verrill maintained to the end of his life the importance of taxonomy as a necessary preliminary to this more specialized biological work.

The successive stages through which modern zoological discipline evolved, such as section-cutting, egg-shaking, transplantation and artificial breeding, had no appeal for him. Not that he failed to follow with some interest the work of the experimental zoologists, although he often charged them with attempting to interpret nature from artificial rather than

natural conditions. Partly for this reason and partly because of his retiring habits he was little known to the younger generation of zoologists, and the extent and importance of his work has not yet been fully appreciated.

During the summer of 1861, while still a student at Harvard, Verrill and three companions made zoological and paleontological collections along the coast of Labrador and at Anticosti Island, regions which up to that time had been but little explored zoologically. The results of his studies on the natural history of Anticosti were admirably recorded by him in a series of papers published mainly in the Proceedings of the Boston Society of Natural History.

For ten years after coming to Yale, Verrill acted as a curator of the Boston Society of Natural History, and during the years 1868 to 1870 he served as professor of comparative anatomy and entomology at the University of Wisconsin, going to Madison each Spring to deliver a course of lectures, in much the same way that our exchange professors visit other universities at the present day. He spent several summers as a geological expert in the employ of various iron and coal mining companies, and is said to have had remarkable success in the location of productive properties. His interest in geology continued as an avocation throughout his life and in 1902 he published a rather extensive report on the physiography of the Bermuda Islands[1] and another on their geological formations.[2] He also taught physical and historical geology to large classes of students in the Sheffield Scientific School from 1870 to 1894. This was done, however, in a somewhat perfunctory manner, and there is little doubt that it was a mistaken policy on the part of the Scientific School authorities to allow so able an investigator in his special field to carry so much routine work in an alien branch of science, even if his classes in zoology were small.

In his zoological activities Verrill gradually concentrated his efforts on marine invertebrates, with particular emphasis on the polyps and echinoderms, or radiates, as they were then called. Collections of corals and other coelenterates from many parts of the world were sent him for study and determination, and on these groups he did some of his most intensive work in later years.

[1] An abridged bibliography may be found at the end of this article, to which references are introduced in the text.

He published more than fifty papers and monographs dealing wholly or in part with corals and other coelenterates and as many on the echinoderms. Outstanding papers on the former group include: Revisions of the species found on the east coast of the United States;[3] synopsis of the polyps and corals of the North Pacific exploring expedition;[4] numerous papers on the Radiata sent to the Museum of Yale College from various parts of the world, and particularly from Brazil and the west coast of America, during successive years;[5] additions to the Anthozoa and Hydrozoa of the Bermudas;[6] Variations and nomenclature of Bermudian, West Indian and Brazilian reef corals;[7] Comparisons of coral faunae;[8] Corals of the genus Acropora;[9] Alcyonaria and actinaria, including Alcyonaria and Actinaria of the Canadian Arctic Expedition.[10] These, together with his extensive monograph on the Alcyonaria of the Blake expedition,[11] place on a secure basis the taxonomy and systematic relationships of the groups concerned.

Verrill's work on the echinoderms is of no less far-reaching importance, and most of his conclusions as to the classification of the serpent stars and starfishes are likewise generally accepted by morphologists. Of particular merit are his later revisions of the taxonomy of the genera and species of starfishes;[12] his discussion of the evolution and geographical distribution of starfishes,[13] West Indian ophiuroidea and asteroidea;[14] and especially his monograph on the shallow water starfishes of the North Pacific, in two volumes, with more than a hundred plates.[15] Verrill also gave much attention to the mollusks, and with the coöperation of his assistant, Dr. Katherine J. Bush, published a number of extensive papers describing numerous new species in this group.[16] Of particular importance were his studies on the cephalopods,[17] especially the giant squids, some of which were more than forty feet in length, and of which he not only had the opportunity of studying a number of specimens, but also, with the assistance of J. H. Emerton, constructed a life-size model, copies of which are to be seen in most of the larger museums of the world. The Pacific coast octopus was similarly modeled.

Perhaps the work which has brought Professor Verrill the widest recognition is the Report on the Invertebrate Animals of Vineyard Sound and Adjacent Waters, published in the Report of the Commissioner of Fish and Fisheries, for 1872

(452 pp., 38 pls.). This stands as a monumental work in that it was the first extensive ecological study of the marine invertebrates of the inlets, tide pools, mudflats, estuaries and other physical features of the southern New England coast. It includes the natural history of the sponges, hydroids, echinoderms, worms, crustacea, mollusks and other invertebrates found in each of these habitats, in so far as they could be learned at the time. In these studies he was ably assisted by his brother-in-law, Professor Sidney I. Smith, whose death after thirty-one years of service as professor of Comparative Anatomy at Yale University was recently recorded in this Journal. For more than thirty years this Vineyard Sound Report was the standard book of reference for all students of the seashore life of the region, and even to-day it is still much used. It is a remarkable fact that for no other region of the American coast has a similar report been published. For the Vineyard Sound region the ecological work started by Verrill and Smith has now been carried to a much higher degree of perfection with the coöperation of a score or more of specialists. It is but natural that the results obtained by this modern practice are vastly more detailed than could be hoped for by the pioneer methods of earlier days.

The field in which Verrill achieved his greatest distinction was opened by the United States Fish Commission. Under the direction of Spencer F. Baird there was inaugurated what was considered in those days a thorough oceanographic study of the coastal waters of the eastern United States, with the object of determining the environmental relationships of the Atlantic fisheries. In 1871, Verrill was placed in charge of the zoological investigations and from that time until 1887 received at frequent intervals the invertebrates obtained from continuous dredging operations. Large numbers of animals representing not only previously unknown species and genera but even new orders thus came into his hands.

Instead of distributing the different groups to other zoologists, Verrill elected to handle them all. From time to time as particularly interesting animals came to hand, he published brief diagnostic descriptions of them and thus became the authority for a great number of new forms. Many of these were first described in this Journal, with more detailed descriptions and figures in the annual reports of the Commissioner of Fish and Fisheries for 1872 (38 pls.), 1873, 1879, 1880 (4

pls.), 1882 (5 pls.), and 1883 (44 pls.) From 1883 to 1886 he had the able assistance of Dr. James E. Benedict, who sorted and provisionally classified the great bulk of the collections, thus making relatively easy the recognition of species new to science.

After the death of Professor Baird in 1887 these dredging operations were discontinued and Verrill began a summary of the results. Taking up the collections group by group he planned a series of monographs to cover each of the groups of marine invertebrates of New England and adjacent waters. The nemerteans[18] and the planarians[19] were thus completed, while the annelids and other groups remained as unfinished manuscripts at the time of his death.

Having described the more remarkable species of invertebrates found on the New England coast, Verrill turned to other fields which might prove more productive. He naturally selected the Bermuda islands, whence he had received so many of the corals which he had studied.

In 1898 and 1901, and again in 1916, he spent some months at the Bermudas. In 1898 he was accompanied by three of his students, and in 1901 by his son as artist and photographer.

A large number of marine invertebrates were collected and studied, sufficient material being secured to require several years for its investigation.

The breadth of his research on the Bermuda islands is indicated by the title page of the first of the two fully illustrated volumes which he later published, the title being "The Bermuda Islands; an account of the scenery, climate, productions, physiography, natural history and geology, with sketches of their discovery and early history, and the changes in their fauna and flora due to man." This volume covers 548 pages, with 38 plates and 250 text-figures.[20] The second volume, on the geology and marine zoology of the islands, is of about the same extent. The separate papers which comprise these volumes were first published in the Connecticut Academy of Arts and Sciences and subsequently issued as a private enterprise of the author.

The sponges, the corals and other coelenterates, the land snails and slugs, the insects, myriopods and arachnids, the crustacea and pycnogonida, the echinoderms, the tunicates and molluscoidea, and other groups were studied in detail and many

species not previously known to the islands were recorded and many others described as new to science.

Among his last publications are three monographs on the crustacea, the macrura,[21] anomura and brachyura,[22] while he left in the hands of the editor of the Geological and Natural History Survey of Connecticut a manuscript of about 700 pages, with 100 plates, on the higher crustacea of southern New England.

For several years prior to 1890 his work on the marine invertebrates was interrupted while he worked on Webster's International Dictionary. To Verrill stands the credit for the excellence of all the zoological definitions and their accompanying illustrations in this monumental publication. He also coöperated in the supplement to the dictionary, published in 1900.

Verrill's publications extend over a period of about sixty-five years and include more than 350 titles on zoological and geological subjects. That he was able to accomplish so much and in such a wide range of subjects is due not only to his inherited mental ability but also, and in large measure, to his most unusual physique. A man to whom illness or even fatigue was almost unknown previous to his eighty-seventh year, he was capable of a prodigious amount of labor, both mental and physical. He was able to keep at the most arduous tasks for many hours a day; in fact it was known to those most closely associated with him that he sometimes worked right through from one day to the next, with at most a brief nap in the early evening. Habits of labor that would speedily have wrecked the life of an ordinary man appeared to contribute to his well-being. But he never used artificial stimulants.

Sleep to him during periods of his most active labors seemed to be in large measure a casual indulgence, taken, quite frequently, while fully dressed and reclining in his desk chair or on a couch conveniently placed in his study. An after-dinner nap from perhaps eight o'clock until nine, then, absorbed in study, writing or drawing, and quite oblivious of time, he would sometimes work until near daybreak; then a little sleep on the couch before breakfast and off to his laboratory. The noon-day meal was often forgotten, while his reluctance to leave his work frequently resulted in his being from two to four hours late to dinner. Not rarely also the lecture hour passed by unnoticed.

A man of large stature, with his massive head covered with abundant locks of wavy hair, and with piercing blue eyes, he made a striking figure in any company. Genial and kindly when he could be persuaded to indulge in social affairs, his self-contained disposition and retiring nature allowed him to make but few intimate friendships.

So marvelous was his memory concerning scientific matters (and yet so poor regarding his personal appointments) that he was able to recall the name and distinguishing characteristics of nearly every one of the thousand and more species of animals which he had described as new to science. He was a skillful artist, and had such powers of visualization that with a stubby bit of pencil he could make a satisfactory drawing of almost any species that he had ever seen. And finally, his ability to quote references to the literature of the groups on which he worked was truly astonishing. Such an encyclopedic mind not only guarded him against the duplication of generic and specific names already applied, but enabled him to disentangle confusions in synonymy which were baffling to others.

Even when most of his former contemporaries were either dead or living in retired leisure, Verrill's eagerness for discovering new forms of animal life continued unabated. At the age of 85, still sturdy and vigorous, and with eyesight that required no glasses for the keen vision of nearby objects, he entered upon the exploration of the marine fauna of the island of Kauai, in the Hawaiian Island group, with all the enthusiasm he had felt in his college days at Anticosti island. During his two years on Kauai he collected nearly a thousand lots of marine invertebrates, writing diagnostic descriptions of many of the species recognized as new to science and making notes on the colors and habits of others. One paper on these investigations was prepared for publication.

After bringing this collection back to New Haven, Verrill suddenly realized that his strength would be insufficient for its complete study. Although in his eighty-eighth year, up to that moment he had been sanguine of much further work, but in a few weeks he was dead.

Verrill was one of the early members of the National Academy of Sciences. He was for some years president of the Connecticut Academy of Arts and Sciences, a fellow of the A. A. A. S., a corresponding member of the Societé biologique de France, and a member of many American learned

societies. He was given the honorary degree of M.A. at Yale in 1867. In 1899 he was lecturer at the Lowell Institute in Boston.

He married, in 1865, Flora Louisa Smith, a sister of Professor Sidney I. Smith, of Yale. Mrs. Verrill died in 1915. Four of their six children are living.

With the exception of the protozoa, the taxonomy of every one of the invertebrate phyla shows the effects of Verrill's labors. In some, the general scheme of classification was modified, in others new genera and species were added. In all, he exhibited what seems to have been a natural intuition as to the significance of morphological characters which amounted almost to genius. It is natural that in the thousand and more species which he described as supposedly new to science, a few are now known to have been previously described from other parts of the world and a few others are now classed as mere varieties, but the marvel is that he could have accomplished such an enormous task with so few errors of judgment and with still fewer errors of observation.

There seems little doubt that future generations will accord him recognition as one of America's greatest systematic zoologists and one of the most productive of our zoological pioneers.

The portrait which accompanies this notice is from "A Century of Science of America," published in 1918 by the Yale University Press, to which acknowledgments are here made.

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TESTIS GRAFTING IN TADPOLES OF RANA TEMPORARIA L. AND ITS BEARING ON THE HORMONE THEORY OF SEX DETERMINATION

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TWENTY FIGURES

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INTRODUCTION

Remarkable progress has been made in recent years in genetic analysis as well as in the field of the physiology of development. The central problem, in which these branches of experimental biology have to meet one day, is that of the immediate cause of differentiation, often designated as the determination problem. In most animals and plants the first cleavage cells and even the cells of more advanced stages contain full sets of chromosomes. We must assume, therefore, that they have the complete series of mendelian genes also. Nevertheless, up from a given stage they differentiate

in divergent directions. Genetic work of the last fifteen years has revealed the existence of a complicated pattern in the arrangement of genes in the chromosomes. It is obvious, however, that there is no direct relationship between localizations in the chromosome maps and determination of the final topography of the body.

Some workers, much impressed by the success of modern biochemical investigation, consider the genes as catalysts or enzymes. These are supposed to produce the visible morphological effect in an indirect way by means of the elaboration of hormones or similar chemical substances with highly reactive properties. There is undoubtedly much striking coincidence between the suggested hypotheses and some of the known facts. However, no conclusive proof has been given for the real existence of any one of the proposed constituents of the chain of chemical processes connecting genetic constitution and morphological differentiation.

The experiments described in the following pages were undertaken with the intention of testing especially the question whether or not hormones are concerned in the process of sex determination.

ACKNOWLEDGMENTS

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ment for work and for so many suggestions received in discussing questions in which we were mutually interested.

Acknowledgments are also due to Mr. K. Toda for his assistance in drawing the curves of figures 1 to 3.

MATERIAL AND METHODS

The animals used belonged to the European species Rana temporaria. Adult frogs were collected near Freiburg (Germany) during their hibernation and carried to New Haven, Connecticut, in February, 1926. In March and April, they were breeding, giving rise to the offspring used in these experiments.

Pieces of adult testes were engrafted into tadpoles before sexual differentiation. A first set of larvae were anaesthetized in a 0.03 per cent solution of chloretone. Five out of fourteen individuals did not recover. Much better results were obtained later on by the use of a new anaesthetic (not yet on the market) received from Sandoz' chemical factories, Basel. The body wall of the larvae was perforated with the aid of sharp needles and iridectomy scissors. Then a piece of adult testis measuring about 1 mm. in each dimension was pushed into the peritoneal cavity. The pieces obviously were relatively large with respect to the size of the larvae. Homoplastic grafts were performed exclusively.

Two sets of larvae of somewhat different age and size were used for the operations.

First set. Fourteen larvae, fifteen days old, 22 mm. total length, 8 mm. body length.

Second set. Thirteen larvae, twenty-seven days old, 30 mm. total length, 11 mm. body length, 1.5 mm. length of hind legs.

Five larvae of the first lot died in anaesthesia, two were lost by accident. Seven were preserved for detailed examination:

Ti 1, tadpole, thirty-three days old, 18 days after operation, 32 mm. total length, 11 mm. body length, 2 mm. length of hind legs.

Ti 2 to Ti 7, metamorphosing frogs, forty-eight days old, thirty-three days after operation.

Out of the second lot three larvae died, while ten were secured for final study:

Ti 17, larva near metamorphosis, thirty-eight days old, eleven days after operation.

Ti 14 to Ti 16, metamorphosing frogs, forty-five days old, 18 days after operation.

Ti 8 to Ti 13, frogs about two weeks after metamorphosis, fifty-six days old, twenty-nine days after operation.

The first lot metamorphosed about four days later than the second one. This rather insignificant difference, without any doubt, is due to a slightly higher temperature in the second aquarium.

It follows from the above data that there was no selective mortality.

The animals were preserved in Zenker's fluid and the grafts as well as gonads of the hosts were cut into sections of 10 μ.

CONCEPTION OF THE EXPERIMENT

In previous studies ('14, '25) it has been established that there exist two groups of geographical varieties of Rana temporaria: the 'differentiated type,' in which at an early stage males and females in equal numbers differentiate out of the indifferent larvae, and the 'undifferentiated type,' characterized by marked protogynous hermaphroditic tendencies. The females of the latter type are not infrequently found to change into males after having reached sexual maturity. On the other hand, the males without exception develop first as females. Under normal conditions, their ovaries do not transform into testes earlier than some months after metamorphosis. At the time of metamorphosis, therefore, we find only one type of animals: all possessing well developed ovaries of the same size and structure and being provided with primordia of oviducts.

In figures 1 and 2 an attempt was made to give a graphic expression of these conditions. Figure 1 corresponds to the

differentiated races. After the middle of the larval period, sexual differentiation takes place, separating males and females very sharply and permanently within a few days.

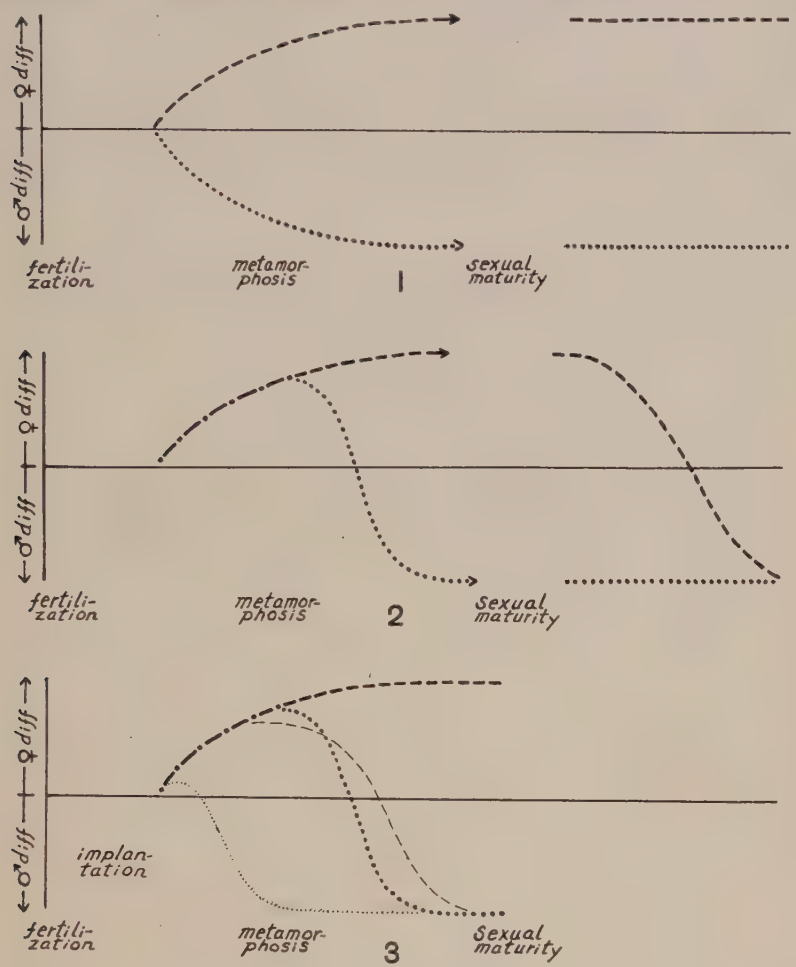

Fig. 1 Diagram describing sex differentiation in differentiated frog races (pure gonochorists). The dash-line corresponds to the genetic female sex; the dotted one, to the genetic male sex.

Fig. 2 Diagram describing sex differentiation in undifferentiated frog races. (protogynous rudimentary hermaphrodites).

Fig. 3 Diagram expressing the expectations as to the effect of early testis implantation upon sex differentiation in undifferentiated frogs, on the basis of the hormone theory. The light lines indicate the respective deviations in both sexes.

The second figure shows how in undifferentiated strains at a corresponding larval period all animals differentiate in the female direction. At the time of metamorphosis they are not in the least different from the females of differentiated races, nor do they show any trace of a segregation in a 1:1 ratio. Sometime after metamorphosis, however, the males separate from the females and their gonads transform into testes. Furthermore, it is shown that adult females manifest a capacity or a tendency to change into males also.

In former experiments of the writer the Freiburg frogs had proved to be an undifferentiated race. They behaved in strictly the same way when raised in New Haven, as the 222 normal control animals of this experiment were all of the female type (preserved during metamorphosis). Figure 10 shows the gonads of such an animal in normal position. The cross-section through one of them (fig. 12) gives clear evidence of the characteristic features of young ovaries: the peripheral germinal epithelium with ripening oocytes and the ovarial sac enclosing the secondary genital cavity. There is but little variation in the size of the 444 gonads which are pure female without exception. The whole of the offspring are completely uniform. In the experimental lots the grafts were introduced during the first or indifferent larval period. If we suppose them to produce hormones, these would have to compete with the hormones coming from the normal action of the genes (enzymes). As the quantitative difference between female and male hereditary factors is lower in these frogs than in most gonochoristic [1] animals, we have an especially excellent chance to detect even a trace of an influence on sex determination coming from part of the graft. In the first place, we ought to find an earlier transition from the female to the male condition or even a direct development of the males (like those in differentiated races). Furthermore, we should expect to see the females transform into males

[1] The term 'gonochorism' was introduced by Haeckel and designates the condition of separated sexes in opposition to the hermaphroditic one.

also, either during the larval period or shortly after metamorphosis. Figure 3 gives a graphic description of these expectations. This diagram is not merely a speculative dash, but corresponds to the conditions which have been observed in cultures derived from overripe eggs (Witschi, '14, '24; Rich. Hertwig described, in 1921, exactly the same result from his experiments with the species R. esculenta). The fact that in previous physiological experiments the sexual character of undifferentiated frog races has proved to be flexible supports the value of this experiment as a test for the hormonic action of testis grafts.

The experiment has to be terminated shortly after metamorphosis, because later on we would find sex-changing animals even in normal controls, as stated above.

THE BEHAVIOR OF THE TESTIS GRAFTS

1. Appearance at the time of implantation

As the operation was performed only two weeks after the breeding activity, the graft material shows practically the conditions of the testis at the breeding season. For details of the yearly spermatogenetic cycle reference may be made to the author's previous paper ('24) on that subject. In order not to implant seminiferous tubules exclusively, but rete tubules also, central parts of the testis were used. Microscopic study of a piece of this material shows the following arrangement of structures (fig. 4): The lumina of the seminiferous tubules still contain masses of sperm bundles, which, however, show some trace of degeneration. Most of the Sertoli cells are in the process of complete destruction. By coalescence and condensation, their protoplasm transforms into deep staining protein spherules, which are scattered below the sperm layer. Later on, they mingle with the sperms extruded into the rete tubules. Along the walls of the seminiferous tubules there is a continuous layer of archispermatogonia (germinal endothelium). Each one of these germ cells is surrounded by a follicle. The rete tubules consist of more or less cuboidal cells with large and

deep staining nuclei. Stroma or interstitial cells are almost lacking in testes of this stage. Some isolated cells may be found in the spaces between crossing tubules or around blood vessels.

2. The grafts eleven to thirty-three days after implantation

The method of introducing testis material into the body cavity in the above-described manner proved successful in

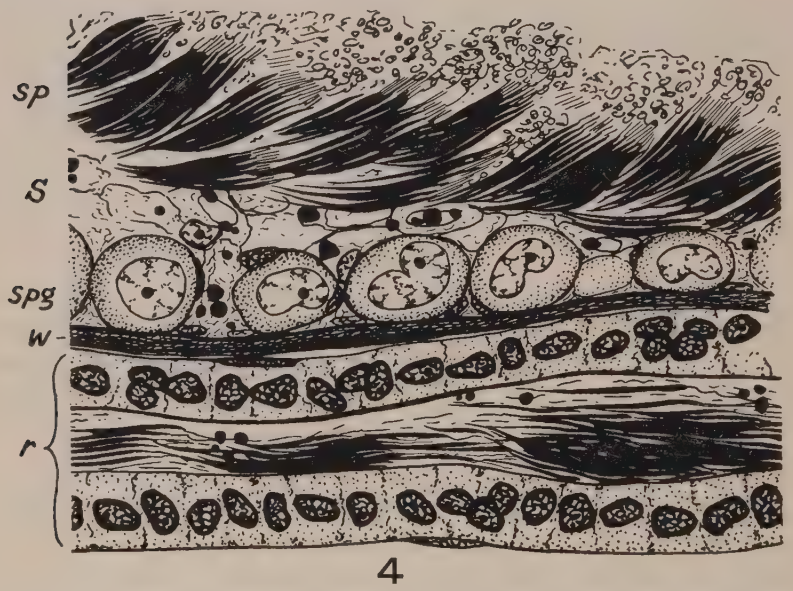

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Fig. 4 Longitudinal section through a rete tubule and half of a seminiferous tubule of the adult frog testis furnishing the graft material. *r.*, rete tubule containing sperm masses; *w.*, wall of the seminiferous tubule; *spg.*, layer of spermatogonia; *s.*, Sertoli cells, most of them in way of degeneration; *sp.*, sperms. × 800.

every one of the seventeen cases. There was a characteristic variation, however, as to the mode of incorporation of the graft. In some cases the testicular particles formed cysts which were connected with the hosts only by stalks (fig. 11). In other cases the graft was in broad contact with some tissues of the tadpole (fig. 17). All grafts were well

vascularized. In the stalked ones abnormally large sinuses were found several times. Most of the grafts were incorporated in the mesentery (nine cases), five grew on the body wall, either attached to the peritoneum or embedded in muscles; three were connected with the liver. It becomes obvious that the latter place is rather unfavorable, as all these grafts show great destruction and nearly complete disappearance of the germ cells. The best growth and preservation of typical testicular structure is found in the mesentery. Seven out of these nine cases are in healthy condition, and even the two exceptional ones do not show abnormal destruction, but merely some retardation, which certainly is due to late incorporation.

The histological changes in the grafts run parallel to a large extent to those taking place in the normal testis after the breeding season. The spermatozoa undergo degeneration. Their long heads break and form globular fragments. At the same time the Sertoli cells enlarge considerably (fig. 19). As the writer has shown in the above-mentioned paper ('24), the Sertoli cells are transformed follicle cells. Here again it is easy to follow their differentiation out of mesothelial cells (figs. 5, 6). The nucleus often includes the large vacuole, which is characteristic for Sertoli cells during the late stages of spermatogenesis (figs. 6, 7). Evidently, they take part in the elimination of the material derived from the break-up of sperms and other cells within the seminiferous tubules. They grow rapidly, accumulate protein spherules and droplets of fat, and as the latter increase rapidly in number and in size the Sertoli cells acquire more and more the nature of luteal cells (figs. 5, 6, 7, 8).[2] During a certain phase of this process, the lumen of the seminiferous tubule is completely filled by the engulfed Sertoli cells. Later on, they decrease again, and finally disappear completely. In the meanwhile

[2] The term 'luteal cells' was prevailingly used for the large fat-laden cells in the ovary only. It becomes obvious, however, that they are homologous to the here-described enlarged Sertoli cells as well as to the interstitial cells (see discussion below). The fact of their morphological identity gave rise to the known interpretations of homosexuality by Steinach and the hen feathering of Sebright cocks by Morgan, both of which proved inconsistent later on.

the spermatogonia multiply until they form thick layers along the inner walls of the seminiferous tubules (figs. 7 and 16). These conditions correspond closely to those found in the normal testis in May and June, with the only difference that in the normal one the Sertoli cells do not hypertrophy to such an extent.

Fig. 5 Changes in the graft during its incorporation. Sperms and some spermatogonia (*spgd.*) degenerating. Sertoli cells and interstitial cells much enlarged. Ti 6. × 800.

Corresponding cellular changes take place outside the tubules (figs. 5, 6, 7 and 19). The few scattered cells of connective tissue and stroma multiply and assume the character of luteal cells, almost like the Sertoli cells. Evidently, there is no specific difference between the various cells of mesodermal origin. In cases of sperm tubules which have their ends cut off, large numbers of Sertoli cells may mingle with the interstitial cells (fig. 18). The former often show more advanced stages of the luteal transformation, staining lighter

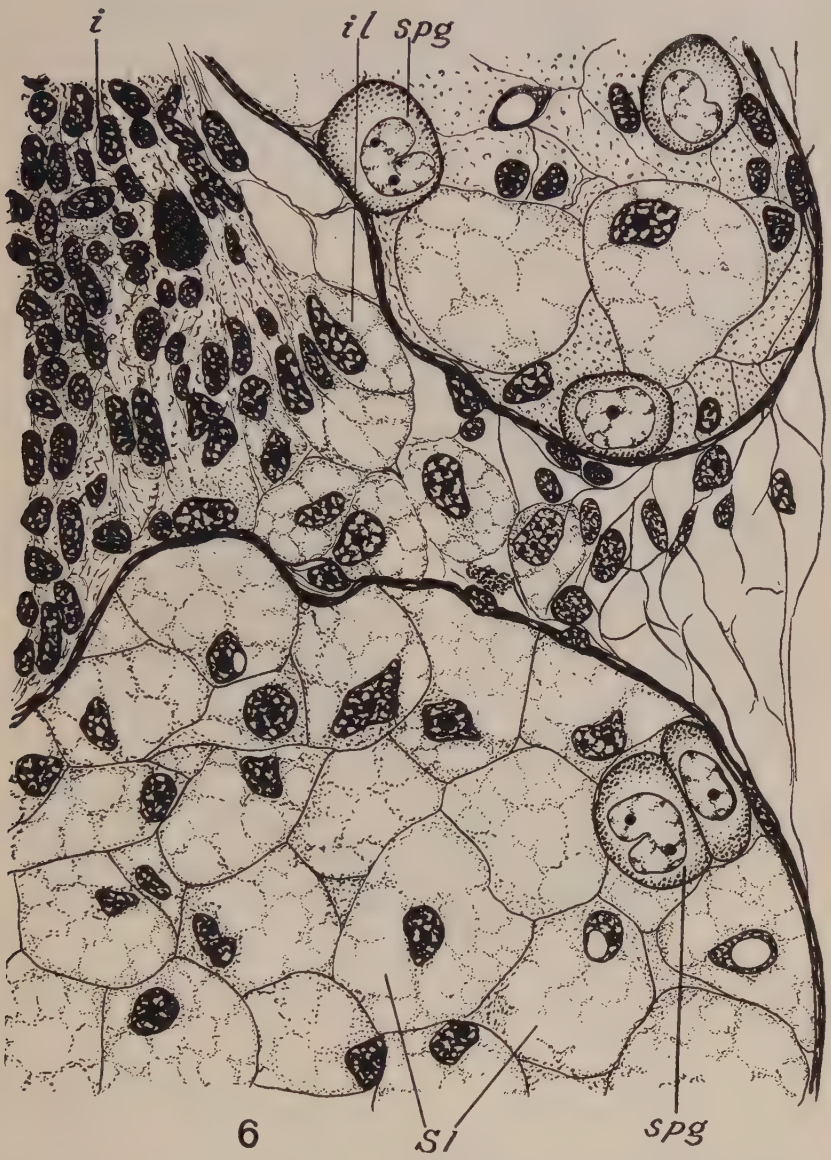

Fig. 6 Section through graft with highly developed luteal cells inside the seminiferous tubules as well as in the interstitium. *spg.*, spermatogonia; *sl.*, Sertoli-luteal cell; *i.*, interstitium; *il.*, interstitial luteal cell. Ti 7. × 800.

than the interstitial cells, but all grades linking both types are abundant also. The fate of the interstitial luteal cells generally is not dissolution, but transformation into fibrous tissue, which becomes very abundant in some of the more advanced grafts.

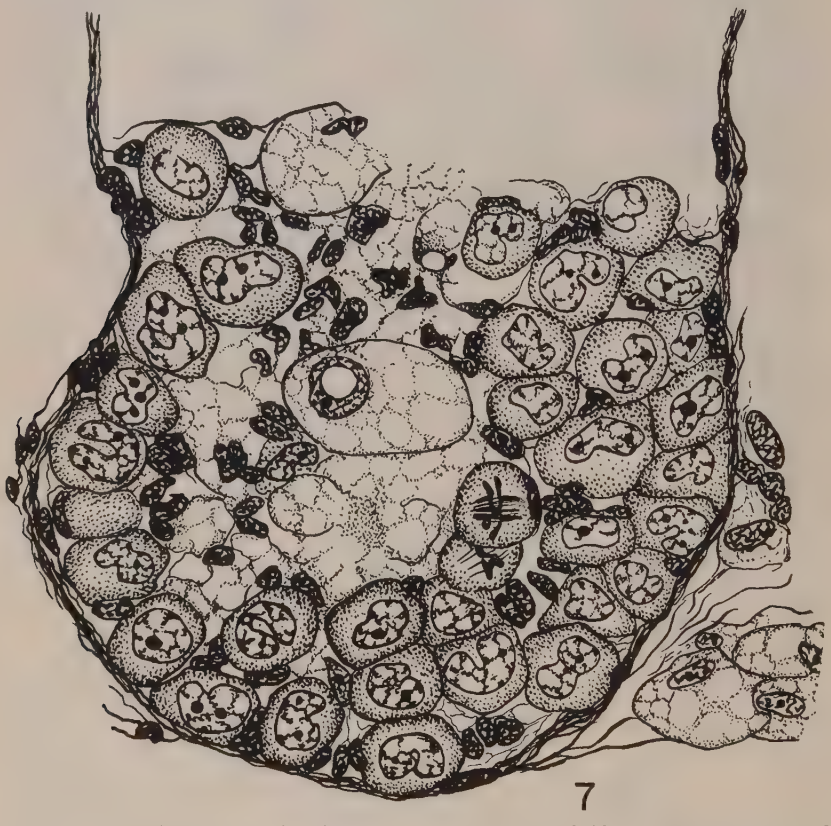

Fig. 7 Detail from graft with well-regenerated seminiferous tubules. Luteal cells much reduced. Ti 2. × 800.

Very often interstitial cells multiply rapidly without acquiring the luteal character. Nodules thus formed (figs. 16, 17) resemble very much the enlarged rete cords of males during the first sex-differentiation process.

It has already been mentioned that the grafts in the liver especially show rapid degeneration of the germ cells. In

such cases the rete tubules are the only well preserved structures, as may be seen from the photomicrograph (fig. 20). The seminiferous tubules either are filled with Sertoli luteal cells exclusively or they may still contain remains of degenerating germ cells, as shown in figure 8. The germ cells as well as the Sertoli cells are detached from the tubular walls. They also are separated from each other in most cases, although germ cells may coalesce and form giant cells. These then are polynucleated, all the more so because each single nucleus

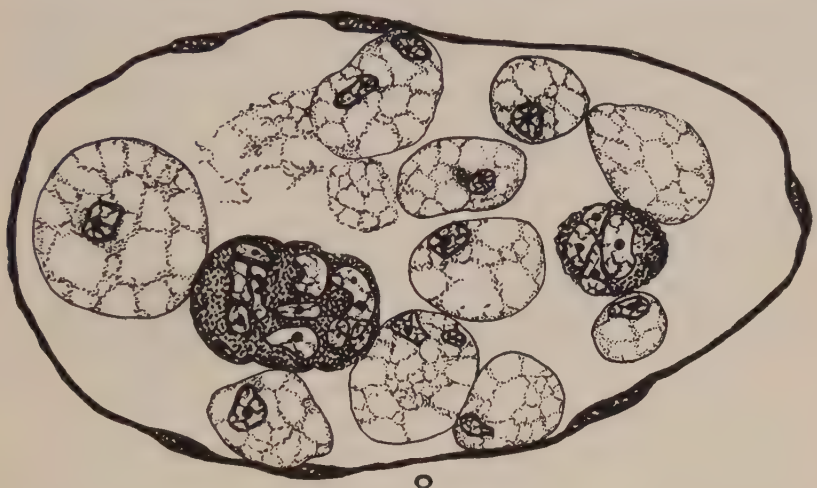

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Fig. 8 Cross-section through a seminiferous tubule of a graft incorporated in the liver. Ti 11. × 800.

divides amitotically into a considerable number of caryomeres. Such degenerating germ cells are always naked, i.e., lacking follicular cells. Similar conditions, as far as germ cells are concerned, have been described by Moore ('24) and Moore and Quick ('24) in regressing seminiferous tubules of mammalian testes.

Grafts in the liver do not equal those attached at the mesentery as to the amount of luteal cells developing out of the interstitial cells. The interstitial cells transform more directly into fibrous tissue, which soon is to constitute the main part of this type of grafts.

THE GONADS OF THE HOSTS

1. The gonads at the beginning of the experiment

Both lots of larvae used in these experiments were still in the undifferentiated period when the implantation was made. The second one, however, consisting of larvae 30 mm. total length, was quite near the beginning of the process of differentiation. Figure 15 shows such a fully developed undifferentiated sex gland of a control larva preserved at the day of operation. The section is through the solid rete cord and shows also the germinal epithelium with the large primordial germ cells. In the first group the same general arrangement is already established, but the sex glands and especially the rete cords are much smaller.

2. The development of the gonads after the testis-implantation

The animals, which were killed at different intervals after the operation, show a typical development of gonads, corresponding completely to that of the 222 normal controls and in agreement with the detailed description of ovary formation given some years ago (Witschi, '14). The results are summarized in table 1.

All animals form ovaries. Animal Ti 1, eighteen days after the operation, thirty-three days old, is in the leptotene stage, i.e., the most advanced germ cells have just reached that stage. Five days later, Ti 17 is found to have large follicles with synaptene oocytes. At the time of metamorphosis the animals Ti 2–7 and 14–16 are at the pachytene stage, exactly coinciding with the controls, which have been preserved at this time. Figure 10 shows the total aspect and figure 12 a cross-section of the gonad of a control animal. The comparison with the corresponding figures 11 and 13 does not reveal any significant difference, despite the presence of a well-growing testis graft (figs. 7, 11, 16) in the latter animal. Two weeks after the metamorphosis (twenty-nine days after the operation), the ovaries are considerably enlarged (animals Ti 8–13). They now contain numerous

TABLE 1

NO. OF ANIMAL	AGE IN DAYS OF		GRAFT INCORPORATED IN THE			THE GRAFT CONTAINS (0, I, II, III INDICATING ROUGHLY THE RELATIVE AMOUNTS)									STAGE OF THE MOST ADVANCED GERM CELLS IN HOSTS OR CONTROLS
	Animal	Graft	Liver	Mesentery	Body wall	Degenerating sperms	Spermatogonia within tubules		Spermatogonia outside tubules		Luteal Sertoli cells	Rete tubules	Interstitial cells		
							Normal	Degenerating	Normal	Degenerating			Indifferent	Luteal	
Ti 1	33	18		+!		III	III	0	I	0	III	II	I	I	Leptotene
Ti 2	48	33		+!		0	III	0	I	0	I	I	II	I	Pachytene
Ti 3	48	33			+!	0	I	0	III	0	0	I	III	I	Pachytene
Ti 4	48	33	+			I	I	I	0	0	III	I	I	I	Pachytene
Ti 5	48	33		+!		0	III	0	I	0	I	II	I	I	Pachytene
Ti 6	48	33		+		III	I	0	0	0	III	I	I	I	Pachytene
Ti 7	48	33		+!		I	II	0	II	0	II	II	III	I	Pachytene
Tk 222	48	—	Control animal												Pachytene
Tk 233	27	—	Control animal												Primordial gonocyte
Ti 8	56	29			+	0	0	0	I	I	0	0	I	I	Auxocyte
Ti 9	56	29	+			0	0	I	I	0	I	0	I	I	Auxocyte
Ti 10	56	29		+!		0	III	I	I	0	I	II	I	I	Auxocyte
Ti 11	56	29	+			0	0	I	I	0	I	I	I	I	Auxocyte
Ti 12	56	29			+	0	0	0	I	0	0	0	I	I	Auxocyte
Ti 13	56	29		+		I	I	I	II	I	I	0	I	III	Auxocyte
Ti 14	45	18		+!		I	II	II	0	0	III	0	I	II	Pachytene
Ti 15	45	18			+!	I	II	II	I	0	III	I	I	II	Pachytene
Ti 16	45	18			+	Graft lost by accident									Pachytene
Ti 17	38	11		+!		III	III	I	I	0	III	0	I	III	Synaptene
			3	9 (7!)	5 (2!)										

!, designates a healthy thriving graft

rapidly growing auxocytes, as shown by the cross-section (fig. 14). It belongs to the same animal (Ti 9) from which the graft represented in figure 18 was secured. It is in every respect characteristic for the entire group.

THE FORMATION OF SECONDARY SEX CHARACTERS

Little attention was paid to the secondary sex characters, as they do not appear until the last days of larval life. At metamorphosis time, primordia of oviducts are regularly

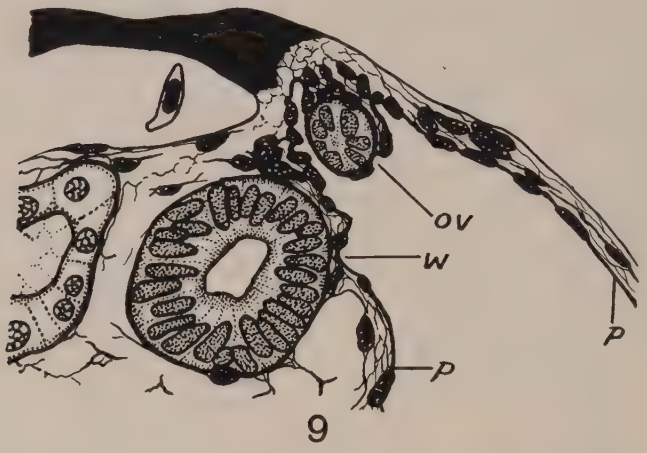

Fig. 9 Cross-section through the lateral border of the kidney and the primordial oviduct of a frog with testis graft two weeks after metamorphosis. *p.*, peritoneum; *ov.*, oviduct; *w.*, wolffian duct. Ti 9. × 800.

found following the lateral borders of the kidneys. Testis grafts do not interfere with their formation, as shown by the camera-lucida drawing of a section through the müllerian duct of the animal Ti 9 (fig. 9).

DISCUSSION OF THE RESULTS WITH RESPECT TO RICH. GOLDSCHMIDT'S HORMONE HYPOTHESIS

It became evident that the presence of testis grafts did not exert the least influence upon the larval and early postlarval development of gonads of R. temporaria. The lack of sex-differentiating hormones thus demonstrated must be due

either to their absolute non-existence or to the accidental non-functioning of the engrafted endocrine gland. The latter condition could conceivably be caused in different ways, and we have to discuss these possibilities.

1. The endocrine system of the graft might have been weakened by postoperative conditions and thus set out of action or rendered insufficient. All attempts to make out what kind of cells found in normal testes might constitute the supposed endocrine gland have failed so far. However, there is no lack of suggestions and we know that practically all components have been taken into consideration: interstitial cells, Sertoli cells, and germ cells. Yet we have described how all these different elements are not only present in the graft, but even may hypertrophy and go through all the stages which ever have been claimed to be the expression of endocrine activity. Furthermore, the grafts always surpass the gonads in size—often by as much as twenty times—so that even a reduced glandular action should exert a definite influence on the host's gonads. A third point to take into consideration is the abundant blood supply, which offers the best conditions for an efficient distribution of hormones. For all these reasons the argument of mere postoperative ineffectiveness is inconsistent. If testicular tissues normally were producing sex-determining hormones, then there is no question but that we should expect to find these grafts functioning in the same sense.

2. Is it possible that the graft was introduced too late, i.e., after the physiological determination of the sex in the host's gonads? It was to meet this possible source of error and to test the time factor in sex determination that two lots of different-aged tadpoles were chosen in these experiments. We have already mentioned that the second group was ready to undergo sex differentiation within a few days after the operation. In the first lot, however, the larvae were but fifteen days old and the indifferent gonads were just formed. Sex differentiation took place fifteen days later, i.e., after the thirtieth day of age. From this alone it would seem probable

that, at least in the first lot, the implantation was not performed too late. The last possible objections vanish when we consider the special nature of the temporaria material. We know that sex is very flexible in this frog and that under normal conditions the males do not differentiate until one or two months after metamorphosis. Thus we know that during the entire larval period sex in these animals is in a highly labile condition, and former experiments as well as unpublished new ones have demonstrated that it is easily subject to changes. For a relatively long period it is a fairly sensitive indicator for sex-determining factors. Thus it becomes perfectly obvious that the testis grafts do not produce any sex-differentiating hormones.

3. Furthermore, it might seem conceivable that the sex-differentiating hormone is elaborated not by the adult testis, but only by the young sex gland, which is just in process of sex differentiation. Our experiments with parabiotic frog twins, joined as early as the late neurula stage, prove this assumption to be untenable (see preliminary paper in Biol. Bull., 1927).

Before generally denying the existence of sex-differentiating hormones, let us consider the facts which led Goldschmidt ('18–'23) to the conception of his hypothesis "that the sex heredity mechanism leads to the production of hormones for the differentiation of the characters of sex." The reading of "Mechanism and Physiology of Sex Determination" is made highly attractive by the lucid presentation of a wealth of most interesting facts gathered from very different fields of the sex problem and their masterly arrangement in accordance with a few logically related principles. However, more and more the facts prove to be complicated and apparently are based upon the action and interaction of a considerable number of determining factors. Moreover, Goldschmidt's attempt to give a common interpretation for such diverse items as sex determination and correlation of primary and secondary sex characters appears unfortunately premature; for when in his book we search for

direct evidence of hormonic sex differentiation, we find only two pieces of work mentioned: the case of the free-martin and Minoura's grafting experiments. Yet from the results of closer examination—published after Goldschmidt's book

Fig. 10 Gonads of metamorphosing control animal. Tk 222. The two white ovaries are situated left and right of the mesentery, on the medial border of the cranial halves of the kidneys. The picture shows also the large fat-bodies. × 13.

Fig. 11 Gonads and graft of a corresponding animal (Ti 2) from the experimental series. The white arrow points to the graft which is attached to the mesentery. × 13.

was written—both cases prove to be not in harmony with the discussed principle.

From the data concerning relatively old free-martins Goldschmidt concluded that the degree of intersexuality is determined by the time when the hormones commence to act, and he expects a complete transformation of one sex into the other if the hormone action begins early enough (pp. 112, 113). More recent work of Lillie ('23) and Bissonnette ('24) extending to younger stages furnishes clear evidence, that even though the fusion of the chorions may be accomplished before sex differentiation, the female cotwin nevertheless develops ovaries. They seem, in the first instance, to be typical female and only secondarily transform into testis-like organs. It is obvious that even in this case of the free-martin there is no evidence of hormon action in the primary sex differentiation.

As to the second-mentioned case, we remember that some years ago Minoura ('21) reported on some atypical features in primary and secondary sex characters found in chicks ready to hatch, after he had implanted testicular tissue in the chorio-allantois membrane of an early embryonic stage. Repetition of this work by Greenwood ('25) and variation by Willier ('25, '27), who engrafted embryonic gonads instead of adult ones, show that Minoura was mistaken in his interpretation. The described atypical variations are found also in normal controls with the same frequency. Willier's experiments are of a special interest with respect to our problem. They show that indifferent gonads are "independent of the host surroundings in the attainment of specific differentiation." From his results Willier derives the conclusion "that hormonic sex differentiating factors are either absent, or, if present, they are ineffective in the modification of the engrafted sexual glands, whether of the indifferent or morphologically differentiated stages of sexual development."

While these two cases were invalidated in Goldschmidt's sense, Burns ('25) reported on a new experiment which would be very much in favor of the hormone hypothesis of

sex determination if his conception proved to be correct. Pairs of parabiotic Amblystoma twins, which were joined on the neurula stage, proved to be unisexual without exception. Burns believes that the sex-determining hormones of the

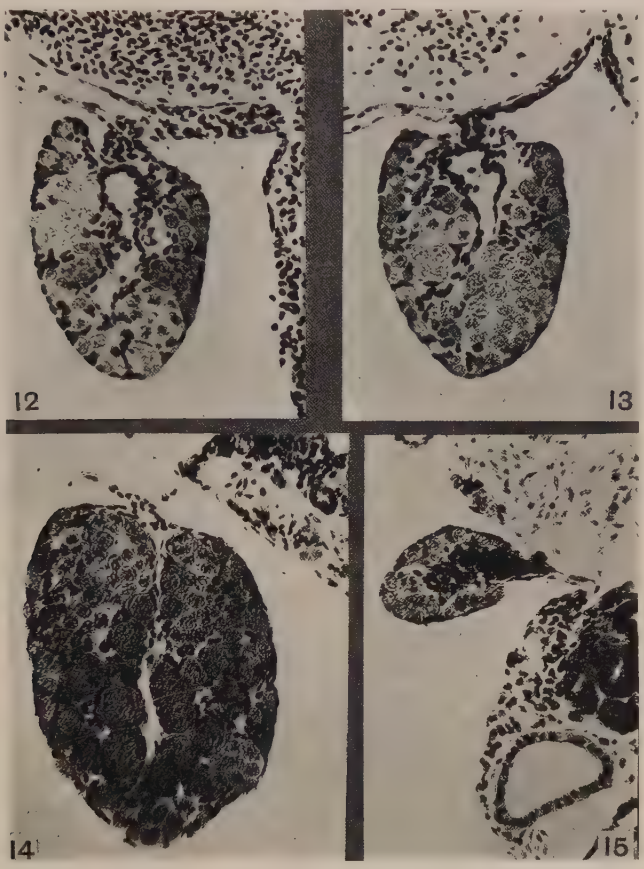

Fig. 12 Cross-section through a gonad of the control animal Tk 222, showing the typical ovarial structures. × 160.

Fig. 13 Cross-section through a gonad of the animal with testis graft Ti 2, showing also a typical female structure. × 160.

Fig. 14 Cross-section through an ovary of the animal Ti 9. Typical for frogs with testis grafts, two weeks after metamorphosis. × 160.

Fig. 15 Cross-section through the undifferentiated gonad of control larva Tk 233. Shows the condition at the time of testis implantation in the second experimental group. × 160.

animal which is somewhat ahead at the critical determinative period are carried by the blood stream in its cotwin and—according to the all-or-none rule—cause it to develop gonads of the same sex. There are some serious objections to such an interpretation. Discussion has to be postponed for a separate paper which will give a more extensive treatment of the writer's own experiments on parabiotic twins. Until this case is settled, it would be inopportune to put forward general conclusions about the physiological mode of sex determination. Notwithstanding, we may point out that, aside from Burns' Amblystoma case, no experimental facts are in favor of the hormone hypothesis, while, on the other hand, there is given full evidence that, at least in chickens and in frogs and, presumably, in cattle also, there is no hormonic factor acting as a determinator in primary sex differentiation.

ON THE NATURE OF INTERSTITIAL AND SERTOLI CELLS

We do not intend to give a full discussion of the still much-confused question of the interstitial tissues in the sex glands, but it appears worth while to pay some attention to the behavior of these cells in the above-described grafts. Everyone who has studied sections through frog testes preserved at different seasons knows that luteal cells are rather infrequently seen. In material fixed immediately after the capture of the animals the writer has found them only during a time between the breeding season and the beginning of the new spermatogenetic cycle, i.e., in R. temporaria during April and May. It is not improbable that fats and kindred substances, which in most vertebrates are stored in interstitial cells, are in amphibians found in the large fat-bodies. In the sections there are generally found but small amounts of tissue between the spermatic tubules, consisting of mere stroma cells. The testis grafts in tadpoles have now revealed that such 'interstitial' cells are capable of rapid multiplication as well as transformation in luteal cells. Under the same condition, the intratubular Sertoli and follicular

cells also changed into luteal cells, and in the case of open tubules the identity of luteal cells arising from both sources was clearly demonstrated. These facts prove that interstitial, follicular, and Sertoli cells are not of different specific character.

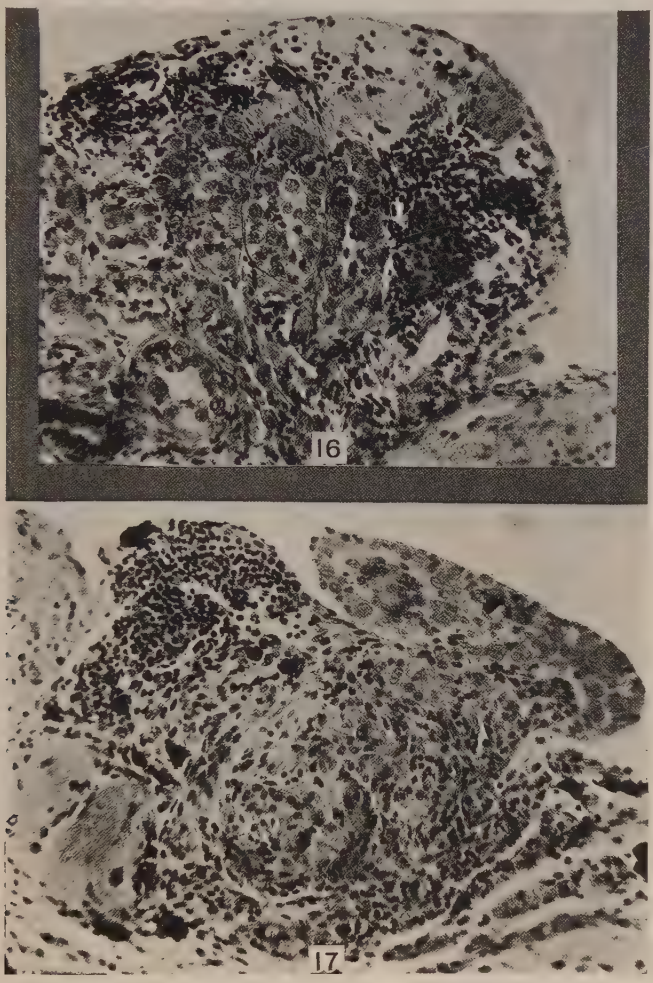

Fig. 16 Section through a part of graft Ti 2 (mesentery), showing regenerated seminiferous tubules and nodules of stroma cells. × 160.

Fig. 17 Section through the graft Ti 3 incorporated in the body wall. Nodules of stroma cells, spermatogonia. × 160.

From the investigations of Seitz ('06), Rasmussen ('18), Nonidez ('20, '22), Sainmont ('06), Winiwarter ('08), Pease ('22), and many others (for literature see Lipschütz, '24) it appears that the lutein cells of the ovary are of exactly the same manifold origin, the stroma and follicle cells being the main source of their formation. The way the follicle becomes a theca and transforms into luteal cells during the atretic processes corresponds closely to the changes in Sertoli cells described above.

Comparison of the life-history thus furnishes evidence of the homology of the luteal cells in both testes and ovaries.

SUMMARY

1. Local races of the European frog Rana temporaria were constant with respect to their peculiar protogynous sex type when bred in New Haven, Connecticut.

2. Pieces of testes from adult frogs were introduced into the body cavity of sexually indifferent tadpoles.

3. The implants became attached in various parts within the body cavity. Though their behavior is somewhat different in the seventeen cases, all the constituents of the testicle prove capable of a normal development.

4. In most grafts considerable numbers of luteal cells were produced.

5. Development and sex determination of the hosts' gonads were in no way influenced by the graft.

6. The theory of hormonic sex determination proves inconsistent.

7. Interstitial cells as well as Sertoli and follicle cells may be converted into luteal cells. This process is correlated with the degeneration and resorption of germ cells.

8. Evidence is given of the homology of the luteal cells in both testes and ovaries.

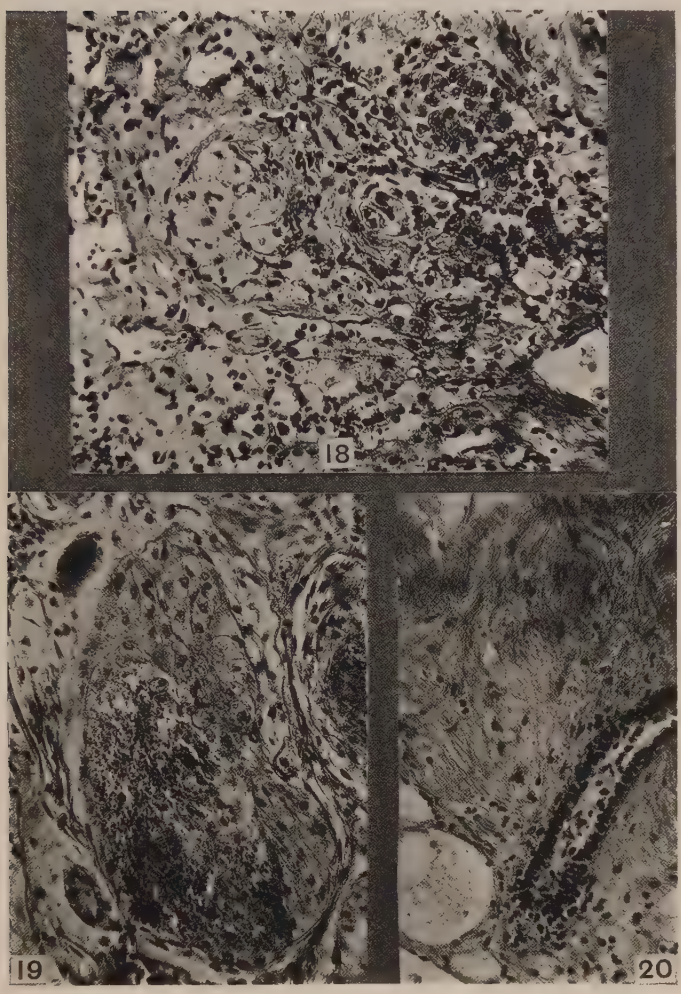

Fig. 18 Part of a section through graft Ti 9 (in the liver) with numerous luteal cells. × 160.

Fig. 19 Cross-section through a seminiferous tubule and the surrounding interstitial tissues of graft Ti 4 (liver), showing degenerating sperms, enlarged Sertoli cells, and stroma-luteal cells. × 160.

Fig. 20 Part of a section through a graft in the liver (Ti 11) with rete tubule, empty seminiferous tubule, and stroma in fibrous transformation. × 160.

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3559

Studies on the Life History of Blepharisma undulans.

LORANDE LOSS WOODRUFF.

From the Osborn Zoological Laboratory, Yale University.

Studies on the life history of *Blepharisma undulans* have been in progress for over five and a half years, all the animals employed being the progeny of a single individual isolated November, 1921. The work was planned to determine, in particular, the viability of this infusorian in the absence of fertilization, and the effects of fertilization on the rate of reproduction, in an attempt to broaden the results from our earlier studies on *Paramecium aurelia*[1] and *Spathidium spatula*.[2] A preliminary statement of the characteristics of this race of *Blepharisma undulans* was presented in 1922.[4]

Whereas earlier workers on this organism found that conjugation occurred rather infrequently and invariably was followed by death, conjugation was readily induced in our pedigree cultures, and the exconjugants proved to be viable in nearly every case. Accordingly excellent material was afforded for the problem at hand.

Two conclusions may be emphasized from the data secured to date:

(1) The animals of this race of *Blepharisma undulans* may reproduce apparently indefinitely without recourse to conjugation; there being no "cycles" in the vitality of the organism, as evinced by the division rate or otherwise, which terminate in death when fertilization is prevented.

(2) Animals isolated from a pedigree line and allowed to conjugate immediately show a markedly higher rate of reproduction than that of the parent line, and this accelerated division rate is

maintained for at least thirty days and, usually, until the experiment is discontinued.

In brief, the conclusions derived from Paramecium[1] and Spathidium[2, 4] that these infusorians are capable of unlimited reproduction, under favorable environmental conditions, without recourse to fertilization may now be extended to Blepharisma; and, furthermore, the conclusion that fertilization accelerates the division rate, as previously demonstrated in Uroleptus[3] and in Spahidium,[2] may now be extended to Blepharisma.

These studies have been aided by a grant from the Bache Fund of the National Academy of Sciences.

This is a preliminary report.

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[3] Calkins, G. N., *J. Exp. Zool.*, 1919, xxix, 121.

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1927, xxiv, pp. 770-771

3560

Inheritance of Atypical Form in Paramecium aurelia.

IMOGENE MOORE. (Introduced by L. L. Woodruff.)

From the Osborn Zoological Laboratory, Yale University.

In all of the lines of a pedigree culture of *Paramecium aurelia,* which had been derived from an animal isolated from the Woodruff culture, in approximately the 12,000th generation, and bred on beef extract in the usual way, there appeared animals showing a "lump" on the peristomal side of the posterior end.[1] During the next week similarly atypical animals appeared in a hay infusion mass culture, which had been seeded with animals from the above lines three months prior to the appearance of the "mutation", and also in the lines of a second pedigree culture derived from an animal isolated from this mass culture.

These atypical animals appeared healthy and active and when isolated each gave rise to two daughter cells showing the lump. As a rule the lump was visible in both daughter cells before division was complete, although in a few cases, it was not apparent until several hours afterward. Furthermore the lumps showed considerable variation in size. With such variations, however, the character persisted in all of the lines, none of them ever again showing a perfectly typical animal.

In the course of time the vitality of the animals became slightly lowered, and at the end of 25 days some of the lines died, having attained from 16 to 24 generations. The remaining lines are still in pedigree, having retained the lump for 30 generations, or 2 months. In the latter lines at least one definite period of endomixis has occurred without any apparent effect on the new character.

In brief, a new character has arisen, in a manner unknown, almost simultaneously in a number of different though related lines

and has been inherited, so far, through 30 generations, involving one endomictic period.

This is a preliminary report.

[1] Woodruff, L. L., *Quart. Rev. of Biol.*, 1926, i, 436; Dawson, J. A., *J. Exp. Zool.*, 1926, xliv, 133.

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New Haven, Connecticut

The Biology of

THE BIRCH LEAF SKELETONIZER

Bucculatrix canadensisella, Chambers

Roger B. Friend

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"Mémoires" shows: "Quand elles sont parvenues à leur juste grandeur, ce qui arrive dans le mois susdit, elles filent contre les feuilles mêmes de trés-jolies petites coques alongées, qui meritent extrèmement d'être connues, à cause de leur figure particulière. Ce sont ces coques qui m'ont déterminé à donner l'histoire de ces Chenilles." He gives a detailed description of the manner in which the cocoon is woven, and also gives brief attention to the pupal and adult stages. There is a plate of illustrations of the larva, the structure of the cocoon, the adult, and the injury to the plant. The species described was *Bucculatrix frangulella* and the host plant, *Rhamnus frangula,* the buckthorn.

In 1832 de Haan published a posthumous volume of Lyonet's works in which there is a description of a "chenille extrèmement petite, mais qui emploie une adresse inconcevable à se filer une coque cannelée." This description formed part of a letter from Lyonet to Réaumur written December 22, 1744, and was later sent to the president of the Royal Society of London to be published if the society saw fit to do so. Most of the description is devoted to the details of the structure and weaving of the cocoon. In his illustrations Lyonet figures the larva, cocoon and its structure, and adult. The larvae were found by Lyonet on the leaves of the oak. This species was *Bucculatrix ulmella* Mann (Zeller).

The history of the genus up to 1862 is given by Stainton in his "Natural History of the Tineina." Linnaeus and Fabricius neglected it entirely, and in 1783 Goeze, in his "Entomologische Beiträge," gave the name *Tinea frangulella* to de Geer's species. Neither de Geer nor Lyonet gave names to the species they described. Retzius, writing contemporaneously with Goeze, and Villers six years later, both gave different names to the *Tinea frangulella* of Goeze. The next person after de Geer to describe a species of this genus was Haworth, who in 1829 in "Lepidoptera Britannica" described *Tinea cuculipenella* with the varieties *beta, gamma,* and *delta.* Stainton notes that although Haworth's descriptions are very vague, *beta* was probably *Bucculatrix boyerella, gamma, B. crataegi,* and *delta, B. ulmella* (Lyonet's species). Three years later, in 1832, appeared the posthumous volume of Lyonet's works, in which is described what proved to be *Bucculatrix ulmella,* as mentioned above. In 1834 Stephens translated Haworth's description of *Tinea cuculipenella* without mentioning the varieties *gamma* and *delta.* In 1833 Treitschke had redescribed de Geer's species as *Elachista rhamnifoliella,* and a new species, *Elachista gnaphaliella.* In 1838 Duponchel figured four species in his "Lepidoptères de France," in the genus *Elachista,* namely, *E. boyerella, E. rhamnifoliella, E. gnaphaliella,* and *E. hippocastanella.* In 1839 Zeller, in "Isis," placed the following species in section A of his genus *Lyonetia: L. rhamnifoliella* (giving reference to de Geer), *L. albedinella* (*boyerella* of Duponchel),

L. hippocastani (*hippocastanella* of Duponchel), *L. cristatella, L. nigricomella, L. cidarella,* and *L. crataegi.*

In 1848 Zeller established the genus *Bucculatrix* with nine species, the descriptions appearing in "Linnaea Entomologica," volume III. The nine species, with the authors credited by Zeller, were:

1. *Bucculatrix cidarella* Tischer
2. *ulmella* Mann
3. *crataegi* Zeller
4. *boyerella* Duponchel
5. *gnaphaliella* Treitschke
6. *frangulella* Goeze
7. *hippocastanella* Duponchel
8. *nigricomella* Zeller
9. *cristatella* F. R.

The species *gnaphaliella* had been previously (1839) placed by Zeller in *Lithocolletis.* He included *Bucculatrix* in a group of leaf-mining moths possessing eye-caps. Much of the history of the genus from Zeller on does not concern us here and will be omitted. Stainton, from whose work much of the above information has been derived, listed in 1862 nineteen species of *Bucculatrix* of which he considered fourteen good and five doubtful. The fourteen were known in the larval form and their food plants were given. Twelve of the fourteen are described by Stainton very fully. This work covers practically all that was known of the genus up to the time of writing.

For the earliest described American species we must turn to the writings of Clemens, who, in the Proceedings of the Academy of Natural Sciences, Philadelphia, for 1860, published the descriptions of four new species of *Bucculatrix: B. coronatella, B. pomifoliella, B. agnella,* and *B. trifasciella.* These descriptions were again published in 1872 in a posthumous volume of the writings of Clemens, edited by Stainton. This volume also includes a description of the genus by Clemens. Chambers, in the Canadian Entomologist, volume V, 1873, described and mentioned nine American species of this genus and stated these to be all the described American species known to him. These nine are: *B. trifasciella* Clemens, *B. capitealbella* n. sp., *B. pomifoliella* Clemens, *B. obscurofasciella* n. sp. (possibly synonymous with *B. coronatella* Clemens),, *B. luteella* n. sp., *B. agnella* Clemens, *B. packardella* n. sp., *B. coronatella* Clemens, *B. thuiella* Packard. Although Chambers considered his *obscurofasciella* possibly synonymous with *coronatella* Clemens, Forbes (1923) gives *trifasciella* Clemens and *obscurofasciella* Chambers synonymous with *packardella* Chambers. It is not proposed to give a discussion of systematics and synonymy here, however. This briefly concludes the history

of the genus in America up to 1875, when the species *canadensiscella* was described.

In the Canadian Entomologist, volume VII, 1875, Chambers described *Bucculatrix canadensisella,* having received his specimen from Canada. This description (see page 401) concerns the adult only and does not mention the larva nor the larval food plants. *B. cidarella* of Europe Chambers considered close to *B. canadensisella,* although quite distinct. The larva of the European species *demaryella* feeds on birch, but according to the description given by Stainton (1862) it also is quite distinct from *canadensisella.*

For twelve years after the description by Chambers there occurs no mention of the species, but in 1887 Lintner recorded the occurrence of the insect in Monroe County, New York, where the larvae were very abundant on the leaves of *Betula lutea* during the fall of 1886. In 1890 Packard recorded what was in all probability this species on the leaves of the white birch at Brunswick, Maine. Lintner again reported it from New York in 1893, this time as injurious to all the native birches in the region of Ausable Forks during September, 1891. The same year Fletcher stated that all the birches around Ottawa, especially *Betula papyrifera, B. lutea,* and *B. alba* (European white birch) were severely injured. From this time on the reports of the insect become more frequent and the injury caused by its larvae more noticed. Hutchings published a brief life history in the 56th Annual Report of the Entomological Society of Ontario (1926), and this treats of the insect more fully than any other publication to date. The species is of some economic importance, and most of the literature on it concerns the injury done to the birch trees.

Systematically the genus has been neglected, and when mentioned it is referred to as aberrant. Forbes (1923) published a key to the species found in northeastern United States with descriptions. For descriptions of species discovered in the present century in America the writings of E. Meyrick, A. F. Braun, and A. Busck should be consulted; and for Old World species see the writings of E. Meyrick, especially his "Exotic Microlepidoptera."

The history of the insect is interesting in view of the fact that at frequent intervals it appears in extraordinary numbers and severely attacks birches over wide areas. In 1886 Lintner found it abundant in Monroe County, New York, and in 1887 it was reported as abundant in Massachusetts. During the years 1890, 1891, and 1892 a serious outbreak occurred in Ontario, New York, and New England. In 1901, 1902, and 1903 it was again very abundant and severely attacked birches throughout this same area. In 1907 a small outbreak occurred on Staten Island, New York, and in 1910 the insect was abundant at Kinderhook, New York.

In 1909 and 1910 birches in Minnesota were extensively skeletonized, and the insect's depredations were severe in Ontario in 1910, 1911, and 1912, and in New England in 1909, 1910, and 1911, growing less serious in 1912 and 1913. The third outbreak of this insect thus covered Ontario, Minnesota, and New England between 1909 and 1912, with small outbreaks in New York in 1907 and 1910. In 1919 the larvae were again beginning to appear in large numbers. This year they were abundant in New Brunswick and were noticed in Connecticut. In 1920 birches were heavily skeletonized and defoliated in Ontario, Quebec, and New Brunswick. In 1921 the infestation continued in these regions, and larvae were abundant in Minnesota and appeared commonly in Massachusetts. In 1922 the injury to birch trees was conspicuous over the Great Lakes region and in New England. This last outbreak began to subside in 1924, although the larvae were injurious in Quebec in 1925. Beginning about 1890 there have been four serious outbreaks of this insect, one about every ten years. Some of the possible reasons for this periodic abundance will be considered under the section dealing with predaceous and parasitic enemies.

III. Systematic Position

The genus *Bucculatrix* was placed by Zeller in a group of minute leaf-mining moths the adult antennae of which possessed eye-caps. Along with *Bucculatrix* were *Lyonetia, Cemiostoma, Nepticula,* etc. The first general treatise on *Bucculatrix* placed the genus in the Tineina (Stainton 1862). It is usually placed in the Lyonetiidae today and is so classified by Forbes (1923). There are, however, differences of opinion as to the classification of Lepidoptera and of this genus in particular. Thus Forbes places *Bucculatrix* in the family Lyonetiidae of the superfamily Tineoidea, but Mosher (1916) places it in the family Bucculatrigidae of the superfamily Gracilarioidea, basing her decision on pupal characters; and Fracker (1915) places it in the family Bucculatrigidae of the Tineoidea. The grouping of families and genera in the Tineina is still apparently an open question. The genus will here be placed in the Lyonetiidae according to the classification of Forbes and considered as slightly aberrant. For a taxonomic account of the genus and a key to the species of northeastern United States the work of this author may be consulted.

IV. Laboratory Methods

The life history data were secured by rearing individual larvae in glass jars or vials, each receptacle containing wet sand and a fresh birch leaf. Observations were made daily. Adults for

oviposition records were caged over a birch twig in a celluloid cylinder with cloth ends. This permitted natural conditions of light and air. The leaves were examined daily with a glass and eggs were marked with a circle of black ink and numbered. Pupae were obtained by simply placing small pieces of heavy cardboard under the plant in a stock rearing cage. The larvae spun their cocoons on the under side of the cardboard. The pupae were kept in a box sunk in the ground until the early summer. Just prior to the period of emergence they were placed singly or in groups of five in glass vials plugged with cotton or in large gelatin capsules, the ends of which were perforated. This made observations on the emergence of adults a simple matter. All life-history studies were made in an out-door insectary. For dissecting fresh material, it was found best to cover the chloroformed specimen with a drop of thick shellac, add one drop of alcohol, allow to set a few minutes, and then immerse in saline solution. The shellac became pitchy and held the insect firmly, but at the same time it could be easily removed from the chitin. For studying the external morphology the insects were boiled in 10 per cent potassium hydroxide until clear and then stained in tetrabromfluorescic acid twenty-four hours. The chitinized plates stained deeply red, and the membranous cuticle a light pink. The body being clear, the internal skeletal structures were readily observed. For the temperature experiments the larvae were kept singly in glass vials or in test tubes, the receptacle in either case being plugged with cotton. The food material was kept fresh and unwilted. The individual insects in all cases were from miscellaneous field collections made in the vicinity of New Haven except where otherwise noted.

V. Morphology

The morphological descriptions will be confined to the external appearance of the various stages and certain important anatomical details of the exoskeleton. The genital organs of the adult will be briefly mentioned as they are of considerable interest morphologically and have more or less influence on the external form. The internal anatomy is not further described here, but it is intended that a description of the anatomy and histology will be produced later.

The original description of the genus by Zeller (1848) is reprinted below.

Bucculatrix Zell.

Elachista Tr. Lyonetia ex p. Zell.

"Caput lanatum, comosum.
"Antennae breviusculae, conchula basali parvula instructae.
"Palpi nulli; os squamis epistomii tectum.

"Alae anteriores caudulatae; cellula discoidales acuta postice venulas 6 emittit; vena subcostalis longissime interrupta; subdorsalis simplex:
"posteriores lanceolatae; vena mediana in 3 ramos divisa, subdorsalis simplex.
"Tibiae posticae pilosae.
"Larva 16 pes supra epidermidem foliorum vivit; metamorphosis in folliculo affixo subit."

The presence of palpi will be brought out later, and the vein which Zeller calls "mediana" in the hind wing is designated in this paper the radius.

The following is the original description of *Bucculatrix canadensisella* by Chambers (1875):

Bucculatrix canadensisella n. sp.

"The ornamentation of this species differs from that of any other yet found in this country, and though allied to *B. cidarella* of Europe, it is still quite distinct.

"Head white. Tuft tipped with dark reddish brown, and the face faintly tinged with purplish fuscous. Upper surface of the thorax brown margined all around with white. Base of the fore wings white, followed by an oblique brown fascia, which is nearest the base on the costal margin, and is followed by an oblique white fascia; all of these are placed before the middle and are followed by a large brown patch which occupies the entire wing to the ciliae, except that it contains a white spot on the middle of the costal margin. The brown patch is margined before on the dorsal margin of the wing by a small tuft of raised brown scales. At the beginning of the dorsal ciliae is a white spot placed a little before, but becomes almost confluent with a longer white costal streak. Behind these streaks to the apex the wing is pale brown, with a darker velvety brown apical spot. Ciliae pale yellowish, with a dark brown hinder marginal line before their middle not extending into the costal ciliae. Hind wings pale fuscous. *Al. ex.* 3/8 inch."

A. Adult

1. External Appearance

As both the above descriptions are rather brief, the external appearance of the adult is here given in a little more detail. By reference to plate XVII and text figure 12 the important markings can be easily followed. Sexual differences are slight and will be referred to in the description.

The general appearance of the adult in repose is shown in plate XVII. The head bears a dorsal tuft of rather long hair-like scales, the center of which is brown and the outer parts white. The "face" is covered with gray or brownish scales. When the insect is at rest the head is bent ventrally so that the labium touches the bases of the prothoracic coxae and the short tongue is curled and concealed between the latter. There are no maxillary palpi, and the labial palpi are very small and concealed beneath the head. The eyes are black and partly concealed by the scapes of the antennae which

are expanded to form eye-caps. These eye-caps are white, and from the anterior border of each there extends down in front of the eyes curved slender scales which give the insect the appearance of having "shaggy brows." The pedicel of the antenna is short, and the flagellum contains 29 segments, each of which bears two whorls of brownish scales. The proximal segments of the

FIG. 12. Adult moth, enlarged about ten diameters.

flagellum have no scales on the ventral side. This "nude" area is usually considered sensory. The first segment of the flagellum (third of antenna) is longer than any of the remaining segments. The antenna is about two-thirds the length of the body and filiform.

The ground color of the fore wings is brown, and although typically reddish, it often varies to a yellowish. The wings are marked with transversely diagonal white bars as shown in figure 12. The basal bar is confluent with a white area on the mesothorax. The second bar forms an angle with the apex directed distally. It sometimes extends completely across the wing and is often interrupted in the center by brown scales. The remaining bars do not cross the wing but terminate near the midline. There are two extending from the costal border and one from the anal border, all three directed slightly apically. Close to the tip of the wing is another white area whose exact size varies somewhat in different individuals. It extends from the costal to the distal border of the wing but does not include the apex, this latter being dark, almost sable, in color. There are two other prominent dark spots on the wing, one at the anal angle and one at the distal margin of the second transverse white bar. Both of these are always present, and sometimes there are other dark spots on the costal border. Beginning slightly distal from the middle of the costal border a row of gray cilia extends around the wing almost to the proximal end of the anal border. The tuft of raised brown scales on the anal border of the wing as described by Chambers is usually conspicuous.

The hind wings are gray and their borders are almost completely ciliated. The superficial difference in shape between the fore and hind wings is due to the more extensive development of scales on the former. The scales on the hind wing are less numerous and do not project beyond the wing borders. Both the wings are really pointed.

The dorsal side of the thorax is brown with white areas laterally, these latter being confluent with the white basal areas on the wings. Each tegula bears a group of eight to ten bristle-like scales which extend along the costal border of the wing as far as the metathorax when the wings are folded. The pleural and sternal sides of the thorax are silvery-white. The coxae are large and of the same general color as the sternum of the thorax except that the lateral borders are brownish, particularly proximally. The femora and tibiae are brown laterally and white medially, as is the first tarsal joint. The tarsal joints two, three, and four each have a white ring proximally and a brown ring distally. The fifth tarsal joint is white, and its scales almost conceal the tarsal claws. At the posterior border of the mesothoracic tibiae at the distal end is a pair of spurs, and a pair of similar spurs is found at each end of the metathoracic tibiae. There is a pair of spines at the distal end of each of the first four tarsal joints. A row of thickly set long hairs is found on the anterior and posterior border of the metathoracic tibiae.

The abdomen is covered with silvery-white scales ventrally and brown scales dorsally. The males have seven segments superficially distinct on the ventral side, the second to the eighth inclusive, and the scales from the eighth practically cover the genitalia. The female has six segments superficially distinct ventrally, the second to the seventh inclusive. Scales from the seventh segment conceal the border between the seventh and eighth, and scales from the latter cover the remainder of the abdomen, giving the appearance of one broad segment. The ninth segment in the female is partly retracted within the eighth, and the tip of the ninth projects very slightly beyond the scales of the latter. The terminal fringe of scales on the male abdomen flares slightly but never does so on the female. The female abdomen is slightly larger than the male. On the dorsal side of the abdomen of each sex there are distinctly demarcated eight segments, the first to eighth inclusive.

The body length averages about three millimeters and the alar expanse seven millimeters. The sexes are of equal size.

2. Head (Text figure 13)

The head is somewhat compressed anterior-posteriorly, and the occipital surface is flat. The antennae are filiform and composed

of 31 joints, of which the first or scape is expanded to form the eye-cap. The second joint or pedicel is short and subspherical. The third joint (first of the flagellum) is half again as long as any of those following. The length of the antennae compared

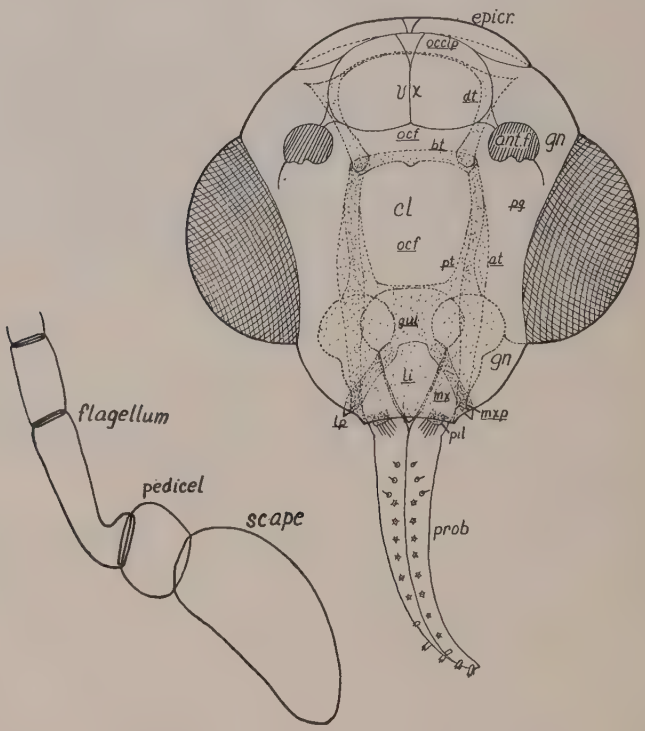

FIG. 13. Head and base of antenna of adult. *ant f*, antennal fossa; *at*, anterior arm of tentorium; *bt*, base of tentorium, *cl*, fronto-clypeus; *dt*, dorsal arm of tentorium; *epicr*, epicranium; *gn*, gena; *gul*, gular region; *li*, labium; *lp*, labial palpus; *mx*, maxilla; *mxp*, maxillary palpus; *occip*, occiput; *ocf*, occipital foramen; *pg*, postgena; *pil*, pilifer; *prob*, proboscis; *pt*, posterior arm of tentorium; *vx*, vertex.

The abbreviations underlined in the figure are on the posterior surface of the head.

with the body length is shown in figure 12. The eyes are black and weakly spherical. There are no ocelli. Between the antennal fossae (ant f) and connecting them is the suture which separates the fronto-clypeus (cl) from the epicranium (epicr). A suture running between the eyes and through the epicranium divides off the vertex (vx) anteriorly. The vertex bears the forward-project-

ing hairs of the dorsal tuft and is divided by a median light suture. The posterior part of the epicranium is likewise divided by a median suture and bears the upward- and backward-projecting hairs of the dorsal tuft. The occiput (occip) lies between the epicranium and occipital foramen and is not sharply demarcated from the postgenae laterally. The fronto-clypeus appears to extend laterally to the eyes. The labrum is not present as a distinct sclerite and is represented by a pair of pilifers (pil) placed one above each maxilla. There are no mandibles. The proboscis (prob) is reduced, being about the length of the head. Each half of the proboscis (the galea) bears on its anterior surface a row of eleven papillate projections which appear pentagonal in cross section and each of which terminates in a short peg. The particular function of these was not ascertained. Near the base of each half of the proboscis and also on the anterior surface are three or four setae. Near the base of each maxilla and projecting from the lateral side is a small protuberance (mxp) which may represent the rudiment of the maxillary palpus. The bases of the maxillae (mx) are, as usual with Lepidoptera, firmly fixed in the ventral (posterior in this case) side of the head. The labium (li) is a small triangular sclerite, with a forward-pointing apex, on the ventral side of the head and lies between the maxillae. It bears a pair of one-jointed palpi (lp). Between the labium and the occipital foramen (ocf) lies a gular region (gul) which is bounded laterally by the maxillae. Its separation from the labium is indistinct. The postgenae are separated dorsally from the genae (gn) by the suture which divides the epicranium and ventrally by the sutures connecting the maxillae with the lower border of the eyes. The genal regions are not distinctly separated from the fronto-clypeus.

The tentorium is similar to that of other Lepidoptera. The body of the tentorium (bt) separates the occipital foramen into a dorsal and ventral part. The anterior arms (at) come forward from the body and then turn ventrally to terminate at the ventro-lateral angles of the fronto-clypeus. The dorsal arms (dt) extend up from the body to the occiput, bordering the foramen laterally. The posterior arms (pt) extend down each side of the lower part of the foramen to the maxillae. In the figure of the head all the abbreviations of the parts on the posterior (morphologically ventral) side of the head are underlined.

3. Cervical Region (Text figure 14)

The head is supported by a pair of laterally placed cervical sclerites (cerv) which extend from the prothorax. At the cephalic end they meet the body of the tentorium, and at the posterior end they articulate with the episterna and then curve medially to meet in the midline.

4. Thorax

The three thoracic segments are distinct, although the prothorax is much reduced. The mesothorax is the most developed, due to the development of the fore wings and the powers of flight. In the following description the nomenclature of Crampton (1909) has been adhered to as far as possible.

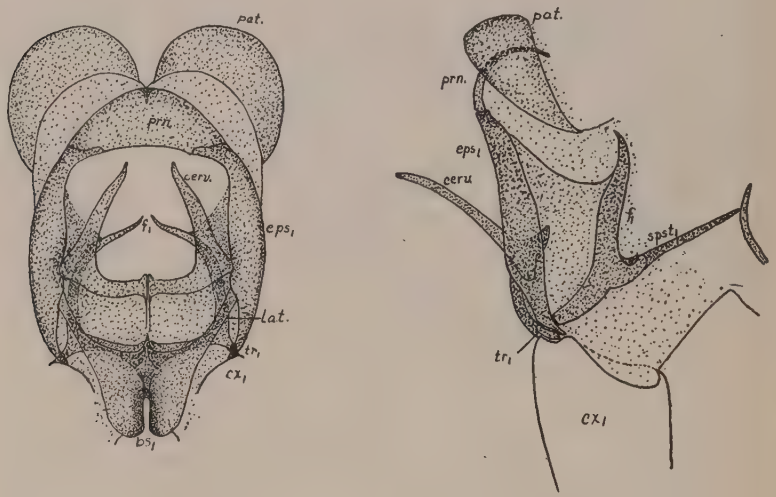

FIG. 14. Prothorax of adult, anterior (left) and lateral (right) aspects. *bs,* basi-sternum; *cerv,* cervical sclerite; cx_1, coxa; eps_1, episternum; f_1, furca; *lat,* precoxal bridge; *pat,* patagium; *prn,* pronotum; *spst,* spini-sternum; tr_1, trochantin.

a. Prothorax (Text figures 14 and 15)

The tergal region of the prothorax consists of a central triangular pronotum (prn) and two laterally placed and conspicuous patagia (pat). The apex of the pronotum meets the prescutum of the mesothorax in the midline.

The pleural region contains one narrow sclerite, the episternum, (eps) which meets the pronotum above and the coxa (cx) below. Anteriorly it supports the cervical sclerites and meets the precoxal bridge (lat) of the sternum. There is a very minute sclerite, the trochantin (tr), at the articulation of the coxa. The epimeron is obsolete. From the posterior border of the episternum the pleural apodeme extends into the body cavity and meets the arms of the furca (f_1). Of the sternal sclerites the basi-sternum (bs_1) is the larger and extends laterally in the precoxal bridge to

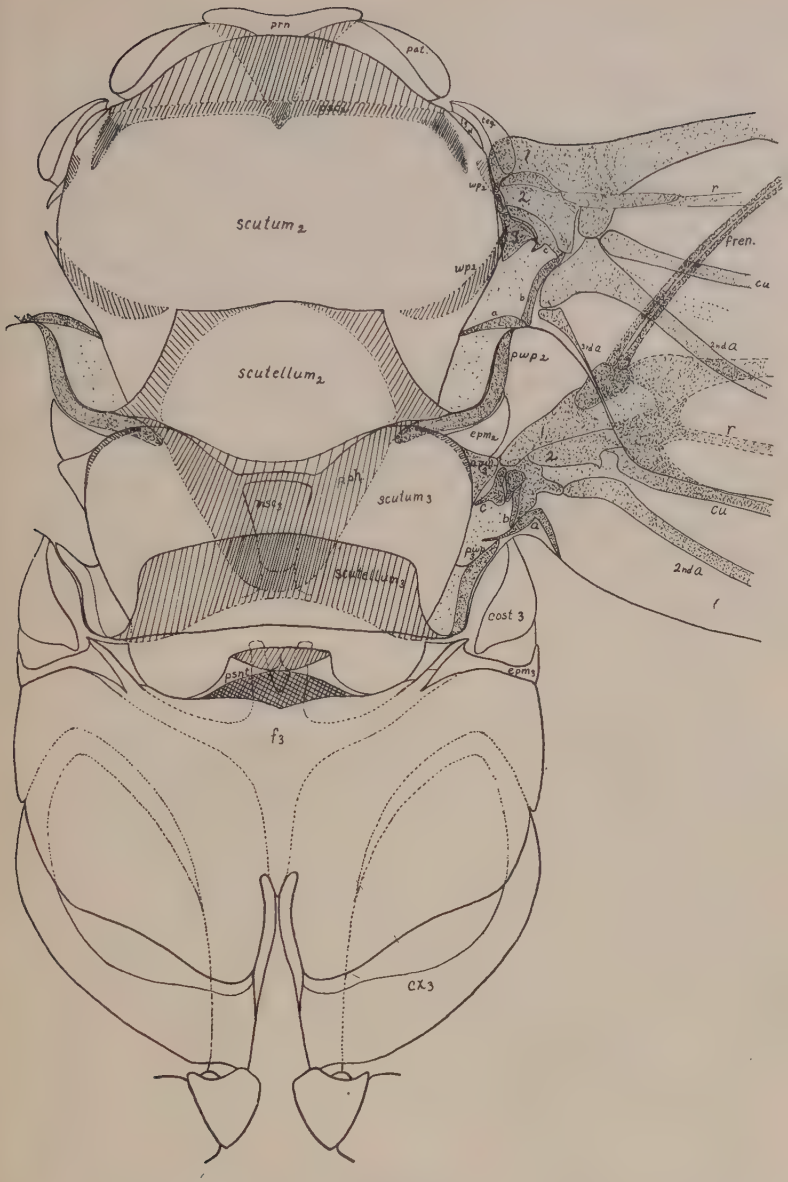

FIG. 15. Thorax of adult, dorsal aspect. *a, b, c,* axillary sclerites; *awp,* anterior wing process; *cost,* costal sclerite; *cu,* cubitus; *cx,* coxa; *epm,* epimeron; *f,* furca; *fren,* frenulum; *msc,* median area of scutum; *pat,* patagium; *pph,* postphragma; *prn,* pronotum; *psc,* prescutum; *psnt,* posterior chitinous plate on metathorax; *r,* radius; *teg,* tegula; *tg pl,* tegular plate; *wp,* wing process; *1, 2,* axillary areas; *2nd a, 3rd a,* anal veins. The inferior numbers indicate the thoracic segment to which the part belongs.

meet the episterna. The median part of the basi-sternum is folded and lightly chitinized, extending slightly down the coxa on each side. The furca-sternum and basi-sternum are not distinctly separated. Caudally from the furca-sternum the narrow spini-sternum ($spst_1$) extends along the ventral midline to meet the presternal sclerite of the mesothorax. What is here called the basi-sternum is apparently the eusternum of other authors. The furca extends up into the body cavity, and from its base a pair of lateral arms extend to meet the base of the episternum on each side.

The prothoracic coxa is not divided into eucoxa and meron, although a faint "suture" extends some distance up from the trochanter along the caudo-lateral surface. The coxa articulates freely with the episternum and trochantin. The trochanter and femur are of the usual type. The tibia bears no spurs. The coxa, femur, and tibia are approximately equal in length, and the tarsus is slightly longer. The basi-tarsus is almost as long as the second and third tarsal joints combined. There are five tarsal joints and each of the first four bears a pair of short spines on the posterior side of the distal end. The tarsus terminates in a pair of claws.

b. Mesothorax (Text figures 15 and 16)

The tergal part of the mesothorax consists mainly of two sclerites, the large scutum (sc_2) and the smaller somewhat rhomboidal scutellum (scl_2). The anterior part of the scutum is rolled in, and from its margin is given off the narrow prescutum (psc_2). This has been considered a true sclerite by some authors, but according to Crampton it is simply a phragma. At its midpoint the pronotum is attached. At the anterior lateral angles of the scutum are the large tegulae (teg) and beneath each tegula is a smaller tegular plate (tg pl). On each lateral side of the scutum are two pointed wing processes (wp_2) which project slightly and which continue medially along the under side of the scutum as chitinous thickenings. These processes help support the wing. The scutellum bears along its anterior and lateral margins a phragma which projects slightly into the body cavity. The posterior lateral angles of the scutellum continue out as the anal borders of the wings. From the posterior margin of the scutellum the large postphragma (pph) projects ventro-caudally into the body cavity. This phragma is made up of a layer from the mesoscutellum and one from the metathorax. The layers are easily separated. There is no true postnotum (or pseudonotum) present as a distinct sclerite. The curved process (pwp_2) which supports the anal area of the wing extends out from the lateral

angles of the scutellum. This is called the posterior wing process by Snodgrass (1909).

The pleuron of the mesothorax is largely made up of two sclerites, the epimeron (epm_2) and episternum (eps_2) separated by the vertical pleural suture. The pleural apodeme extends into the body cavity from this suture. The episternum is divided into a dorsal anepisternum ($aneps_2$) and a ventral katepisternum ($keps_2$) separated by a triangular middle area. At its anterior margin the anepisternum rolls in medially. From the dorsal margin of the sclerite the alar process (alp) projects upward and supports the wing, and the tegular arm (tega) extends anteriorly to the anterior lateral angle of the scutum where it abuts against the tegular plate. The tegular arm and alar process together with a ventral projection on the anepisternum appear to form a single anchor-shaped sclerite fused with the latter and separable from it with no great difficulty. The katepisternum meets the sternum ventrally. The epimeron is a single undivided sclerite somewhat membranous dorsally. It meets the posterior wing process and then arches over as a narrow arm to meet the arm of the furca (f_2). Just under the anal area of the wing and dorsal to the epimeron is the somewhat elongate costal sclerite ($cost_2$). There is no distinct trochantin but it may be represented by a triangular area just over the coxa. The pleural apodeme widens at this region and forms a support for the coxa.

The anterior sclerite of the mesosternum (presternum, pst_2) projects forward from the basi-sternum (bs_2) to meet the posterior sclerite of the prothoracic sternum and extends slightly beyond it into the body cavity. This sclerite widens as it meets the basi-sternum. The latter is triangular, its apex being posterior, and is divided by a median longitudinal suture. From this suture and extending into the body cavity is a median chitinous blade (mbl). Posterior to the basi-sternum is the furca-sternum (fs_2) which extends down the medial side of each coxa as a pedal region (pdr_2) and holds the coxa rigidly to the body. The furca (f_2) arises from the furca-sternum and sends from its base a short curved process (fpr_2) into the body cavity anteriorly. The arms of the furca meet the arms of the epimera dorsally. The latero-sternites extend from the basi-sternum laterally to the pleural suture.

The mesothoracic coxa (cx_2) is divided into an anterior eucoxa ($eucx_2$) and a posterior meron (mer_2) by a vertical suture on the outer side. On the medial surface of the coxa lies a heavily chitinized angular plate (cs_2) which meets the pedal region of the furca-sternum. The leg articulates at the trochanter, the coxa being immovable. The tibia bears at its distal end on the posterior side a pair of spurs of which the outer is longer. The tarsus is

similar to that of the prothoracic leg. The mesothoracic leg is slightly longer than that of the prothorax.

The wing venation (figure 17) is much reduced. The subcosta and costa are probably represented by the single costal vein. The radius (r) is rather faint at the base and gives off five branches distally. The median vein has disappeared except for the branches m_1 and m_2. The cubitus (cu) is single. There is a faint fold (1st a) which may represent the first anal vein. The second (2d a) and third (3d a) anals are distinct. There is some variation in the origin of r_4, as it sometimes branches off distally to the position shown in the figure. The costal vein bears a retinaculum (ret) for the frenulum. The veins named above are according to Forbes (1923).

The axial sclerites of the wing are as shown on the right side of figure 15. The sclerites a, b, c, 3, and the small sclerite between 1 and 3 are hard chitinous plates, but those marked 1 and 2 are thickenings of the wing similar to veins. The alar process of the pleuron abuts on 2, as does the anterior of the scutal wing-processes. The posterior of the two scutal wing-processes abuts on 3, and the posterior wing-process supports a. The anal area of the wing folds along the outer border of b.

c. Metathorax (Text figures 15 and 16)

The scutum (sc_3) of the metathorax is divided medially by a triangular area (msc_3). This does not appear to be a distinct sclerite but simply a more lightly chitinized region. The postphragma of the mesoscutellum is attached to the anterior margin of the scutum, its line of attachment extending to the wing process (awp_3) at the anterior lateral angles. The scutellum (scl_3) is a band stretching across the base of the scutum and appears to overlap the latter, due to the presence of a phragma which projects caudo-ventrally into the body cavity. From the posterior border of the scutellum a membrane drops ventrally to meet a chitinous arm which forms a bridge between the ends of the epimera. The center of this bridge bears a chitinous plate (psnt) to which the tergum of the first abdominal segment is attached. This represents the pseudonotum (Snodgrass), although much modified from a primitive condition. At its lateral angles the scutellum continues into a narrow posterior wing process (pwp_3) which supports the anal area of the wing.

The pleuron of the metathorax resembles that of the mesothorax. The trochantin area at the head of the coxa is more distinct here, however. Dorsally the alar process (alp) continues directly with the pleural apodeme, and the anepisternum ($aneps_3$) bears another process which also supports the wing. The costal ($cost_3$) sclerite is prolonged anteriorly as a long arm. The

epimeron (epm_3) extends further posteriorly than does the same sclerite in the mesothorax.

The sternum of the metathorax differs markedly from that of the preceding thoracic segment. From the central basi-sternum (bs_3) extend the narrow latero-sternites (not shown in the diagram). The basi-sternum extends caudo-ventrally to meet the

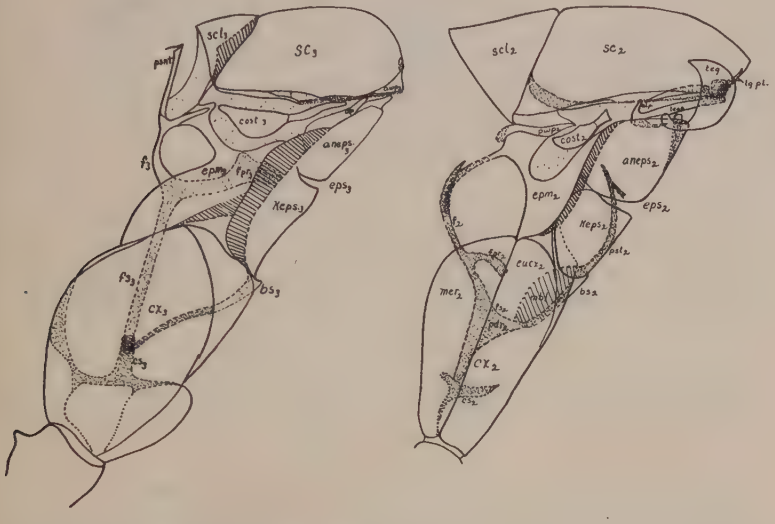

FIG. 16. Meso- (right) and meta- (left) thorax, lateral aspect; *alp,* alar process; *aneps,* anepisternum, *bs,* basi-sternum; *cs,* median coxal support; *eucx,* eucoxa; *fs,* furca-sternum; *fpr,* furcal process; *keps,* katepisternum; *mbl,* median blade; *mer,* meron; *pdr,* pedal region; *tega,* tegular arm; *sc,* scutum; *scl,* scutellum. For other abbreviations see figure 15.

furca-sternum (fs_3) at the coxal support. There is no pedal region of the furca-sternum, but the coxa is held rigidly by this sclerite plus the basi-sternum. The furca-sternum extends as a narrow arm dorsally and then divides into a furca (f_3). The anterior furcal process (fpr_3) is very large and the furca is heavy. Dorso-laterally the arms of the furca meet those of the epimera.

The meron of the coxa is much reduced and occupies a posterior-medial position, only the eucoxa being visible laterally. The tibia bears a pair of spurs on the posterior side of each extremity, and the outer spur of each pair is the longer. The leg is otherwise similar to that of the mesothorax.

The wings (figure 17) show greatly reduced venation. There are, besides the costal, three principal veins, the radius (r), cubitus (cu), and the second anal (2d a), the median being represented

by two branches only. The costal vein probably represents the combined costal and subcostal. The radius is single and from it there branch the two divisions of the median (m_2 and m_3). The cubitus is single. The frenulum (fren) consists of two stout setae that are held in the retinaculum of the fore wing.

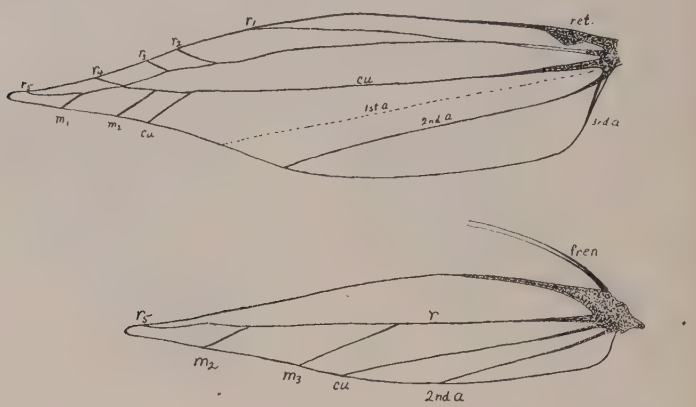

FIG. 17. Fore (above) and hind (below) wings; *1st a, 2nd a, 3rd a,* anal veins; *cu,* cubitus; *fren,* frenulum; *m,* median; 1-5, radius; *ret,* retinaculum.

The axial sclerites of the hind wing are as shown in figure 15. An angular sclerite (a) in the anal region is pivoted on the posterior wing process. The anal region folds along the outer side of this sclerite. Two sclerites (b and c) lie between this and the anterior wing process. These three constitute the chitinous axial plates homologous with those of the fore wing. The areas marked 1 and 2 are thickenings of the wing similar to veins and are homologous to the same areas of the fore wing. The sclerite c may correspond to 3 of the fore wing, and the sclerite b to b and c of the fore wing. The alar processes of the pleuron abut on the area marked 2, and the subcostal area (1) meets the anterior wing process and the sclerite marked c.

Snodgrass (1909) has described the typical arrangement of the axial sclerites in the wing, but the tracing of these in the wing here described is uncertain, due to the difference in arrangement, and hence the letters and numbers as given here do not correspond to those of the above author.

5. Abdomen (Text figures 18 to 20)

The abdomen has nine visible segments in the female and ten in the male, although in the latter sex the tenth is reduced to the socii. The apical segments in each sex are modified to form the

external genital apparatus. The first segment has a strongly chitinized tergum, probably a development in accordance with its function of supporting the abdomen on the thorax. The sternum of the first segment is indistinguishably fused with that of the second and both are quite membranous. The identification of two sterna is furnished by the presence of two spiracles on each side.

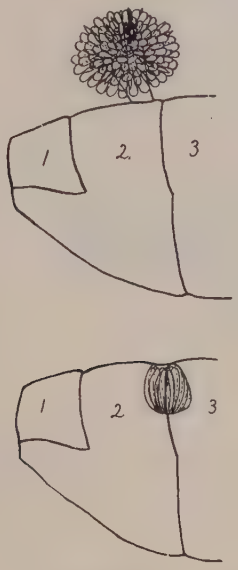

FIG. 18. "Alluring" organ on abdomen of adult male, expanded above, retracted below.

In the female the segments from two to seven inclusive are of the usual unmodified type, but in the male the second segment shows a peculiar sexual dimorphism. On the caudal margin of the tergum of this segment is located a protrusible organ which, for want of a better name, has been termed an alluring gland. Similar organs called alluring glands have been described as occurring on other parts of male Lepidoptera, and until a histological and cytological investigation is made of this particular case, the common term will be used in describing it. In other species of Lepidoptera there is considerable evidence that these organs give off a distinct odor when protruded, but the alluring function of these in a sexual sense is not definitely proved. This "gland" is shown in figure 18 protruded (above) and retracted within the abdomen (below). When retracted it folds in an eversible sac, and when protruded the entire organ, including the sac, projects out from the body, looking for all the world like a composite flower.

The scales composing it are of two kinds, some pointed and some lobular. This organ is found in all males and never in the females. The remainder of the male abdomen up to and including the eighth segment is in no wise unusual. In the female the eighth and ninth segments (figure 19) are modified somewhat. On the sternum of the eighth segment is a slight protuberance

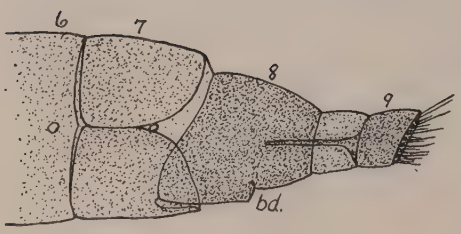

Fig. 19. Tip of abdomen of adult female. *bd,* opening of bursa duct.

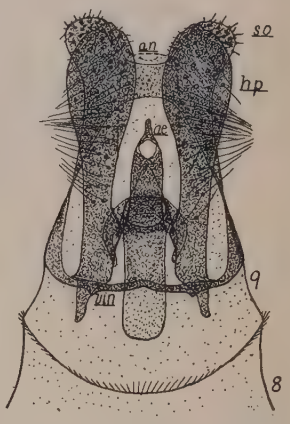

Fig. 20. External genitalia of adult male. *ae,* aedoeagus; *an,* anus; *hp,* harpe; *so,* socius; *vin,* vinculum.

which marks the copulatory opening leading into the bursa duct and thence to the bursa copulatrix. At the end of the ninth segment is the external opening of the vagina (ventrally) and the alimentary tract (dorsally). Petersen (1900) has described in some detail the female and male genital organs of Lepidoptera and shows the transitional stages from the type having one genital opening (at the tip of the abdomen) to that having two as here found. The presence of two genital openings is forecast in the pupa. On each side of the copulatory opening is found a tuft of orange-colored scales, and a third tuft is found on the dorsum at the anterior margin of the eighth segment. These three tufts are normally concealed from view under the posterior margin of the seventh segment. The lateral apodemes from the ninth project back into the eighth segment. The tip of the female abdomen is usually telescoped so that the eighth segment is partly retracted within the seventh, and the ninth is retracted within the eighth. The posterior part of the eighth is membranous. In the illustration the abdomen is shown with these segments drawn out into view. The tenth segment is not developed.

In the male (figure 20) the ninth and tenth segments are much modified and are usually retracted within the eighth. As shown in the figure, they are drawn out to expose the external genitalia. The nomenclature given is according to Eyer (1924). The tergum of the ninth forms a "roof" over the anus (an) which lies just beneath it. It is called the tegumen. Attached to its distal end are the socii (so) which really belong to the tenth segment and form the anal armature. They are paired and bear many short spines and setae. The sternum of the ninth segment consists of a narrow chitinous band, the vinculum (vin), which is fused with the tergum on each side. The paired claspers, called harpes (hp), articulate with the vinculum and are appendages of the ninth segment. They also bear many setae and short spines. The cone-like chitinous organ through which the aedoeagus (ae) projects is called the anellus and also probably belongs to the ninth segment as do the rest of the genitalia. The aedoeagus is a heavily chitinized tube supported by the anellus and tapering to a point distally. The penis itself is a soft eversible tube contained within the aedoeagus and is protruded from the ventral side of the tip of the latter.

There are on each of the segments one to seven inclusive a pair of spiracles, and visible through the ventral wall of the abdomen are the four pigmented abdominal ganglia of the nerve cord. The ganglia of the entire ventral nerve cord of all stages of this insect are deeply pigmented and usually visible externally. In the adult the appendages conceal all but the abdominal, and these are found at the second segment and at the junctions of the third and fourth, fourth and fifth, and fifth and sixth respectively. The last is larger than the others, being a compound ganglion. The third and fourth abdominal ganglia are often contiguous and sometimes are fused to some extent.

6. Genital organs and alimentary tract (Text figures 21 and 22)

The internal genital organs of the male and female are diagrammatically illustrated in figures 22 and 21. In the female the bursa copulatrix (bur) is by far the most conspicuous of these organs, and it occupies much of the anterior part of the abdomen, lying in the region of the third segment. It is connected by a duct to the external opening in the sternum of the eighth segment, and from the dorsal side of this duct near its external end there arises the long slender seminal duct which permits the passage of spermatozoa from the bursa into the oviduct and thence into the seminal receptacle (rec.sem). The common oviduct divides into two ducts (ovid) from each of which are given off four ovarioles (ov), each of which terminates in a filament. The filaments on each side unite with each other. The ovarioles extend from the

oviduct along each side of the bursa to its anterior end, curve dorsally and posteriorly, then dorsally and anteriorly to a common point just above the bursa where the group from each side is attached by the filament tips to the dorsal wall of the abdomen.

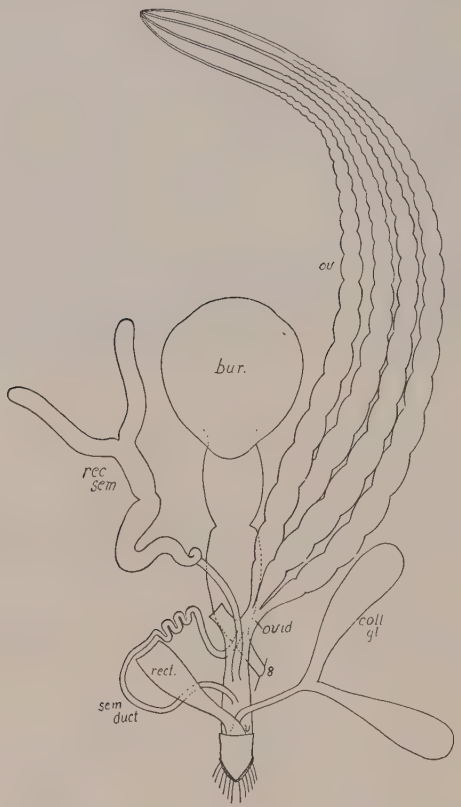

FIG. 21. Reproductive organs of female. *bur,* bursa copulatrix; *coll gl,* colleterial glands; *ov,* ovarioles; *ovid,* oviduct; *rec sem,* seminal receptacle; *rect,* rectum; *sem duct,* seminal duct.

The alimentary tract passes ventrally and to the right of the bursa, curves dorsally to pass above the union of the oviducts, then goes over the common oviduct to the tip of the abdomen, the rectum lying above the vagina. The ovarioles are of the polytrophic type, that is, the nutritive cells alternate with the ova. The seminal receptacle is bilobed and is attached to the dorsal wall of the common oviduct. The colleterial glands (coll gl) are paired and

are connected by a common duct to the dorsal wall of the vagina. They secrete the adhesive substance which attaches the egg to the leaf. In the illustration the genital organs are shown spread out and not in their normal positions.

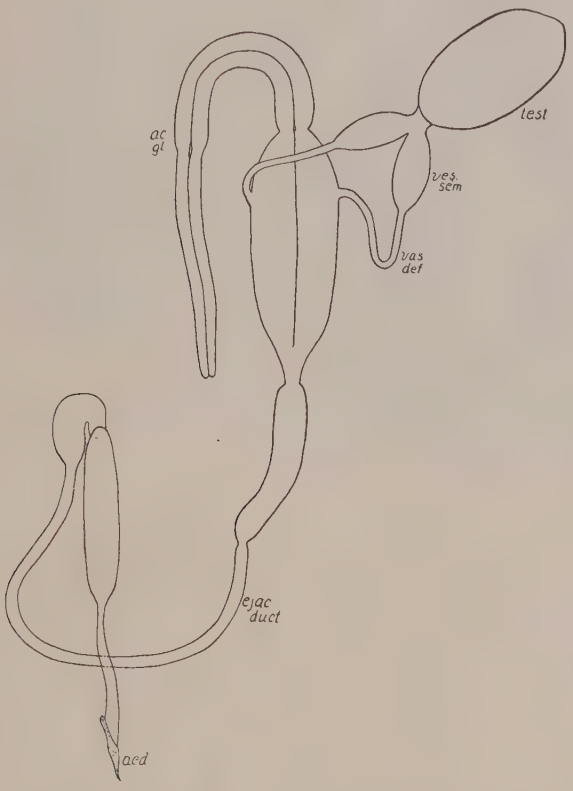

FIG. 22. Reproductive organs of male. *ac gl,* accessory glands; *aed,* aedoeagus; *ejac duct,* ejaculatory duct; *test,* testes; *vas def,* vas deferens; *ves sem,* seminal vesicle.

In the males the testes (test) are united and enclosed in a common scrotum. The vesicula seminales (ves sem) are paired and unite just under the testes. From the vesicula seminales, which are really enlargements of the vasa deferentia (vas def), the latter ducts pass to enlarged chambers which lead to the ejaculatory duct (ejac duct) which in turn terminates in the aedoeagus (ae). The accessory glands (ac gl) which presumably secrete a substance which mixes with the spermatozoa, are paired and

are connected with the enlargements at the terminations of the vasa deferentia. In some insects these glands secrete a substance which forms the spermatheca (especially Orthoptera). They occur here attached to each other rather loosely.

The alimentary tract begins anteriorly in a large muscular pharynx from which two long filamentous salivary glands are given off and extend through the thorax beside the slender oesophagus. The latter extends from the pharynx through the thorax to the mid-gut in the abdomen and gives off a slender tube which enlarges to form a rather large crop. The latter lies mainly in the abdomen over the mid-gut. In the female the mid-gut lies under the bursa and extends to a point between the common oviduct and the bursa. It is much greater in diameter than the pharynx. At the termination of the mid-gut the malpighian tubes are attached, one on each side. These are slender and each branches once. Of the two branches of each tube one divides once and the other remains single. This gives three branches of each tube which lie in the region of the ovarioles. The hind-gut extends from the origin of the tubules and enlarges at its posterior end to form the rectum which terminates in the anus. The crop and mid-gut were empty in all the specimens dissected, which adds evidence to the belief that the adults do not feed. In the male the alimentary tract passes to the right of the ejaculatory duct.

B. The Egg (Text figure 23)

When first laid the egg is a translucent white, but it becomes more opaque after a few days. In shape it is flatly ovoid, and the longest diameter is 0.25 mm. It is made to stick to the leaf by an adhesive substance which surrounds it on the leaf surface in a narrow encircling band. The hexagonal sculpturing of the surface is quite distinct, and the micropyle occurs close to one end. When it leaves the oviduct the egg is soft and flexible, and its shape changes from approximately spherical when it is placed on the leaf surface. The eggs are always laid singly and scattered over the leaf, as shown on plate XVII.

C. Larva (Text figures 24 and 25)

When first hatched the larva is minute (.35 mm. long), translucent, apodous, and flattened, a typical leaf-mining type. When it leaves the mine at the close of the third instar it has assumed a cylindrical form, the head has shifted from its former horizontal plane to a plane nearly at right angles with the body, and all the legs are present and functional. It measures about 2.5 mm. in length. When fully grown (plate XVII) the larva is about 6.0 mm. long and yellowish green in color with the setae on white

tubercles. The prothoracic shield is not conspicuous. The head is brown and typically that of a leaf-eating larva. There are the usual prolegs on abdominal segments, three, four, five, six and ten.

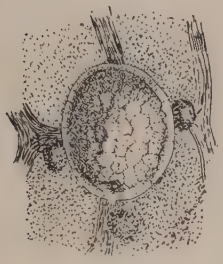

FIG. 23. Egg, much enlarged. Actual longest diameter .25 mm.

FIG. 24. Head of larva of 5th instar, ventral aspect. *ant,* antenna; *mx,* maxilla; *oc,* ocellus.

The radical change in structure which occurs at the second molt is due to the change in feeding habits and environment of the larva, for the third instar comes out of the mine to the leaf surface. This necessitates the acquisition of legs and the shifting of the plane of the head. The dorsal part of the head capsule is longer than the ventral, and this makes the posterior margin of the epicranium in the leaf-mining instars, where the head is horizontal, push back into the prothorax. Trägårdh (1913) has described structural transitions in several leaf-miners which change their feeding habits. The third instar resembles the fourth and fifth in general, but the setal pattern is somewhat different and there are fewer crochets on the prolegs. The prolegs of the abdominal segments three to six inclusive have one transverse row containing two crochets, and the prolegs of the anal segment bear one crochet.

The fourth instar is like the fifth except for size and no further mention need be made of it. The fifth instar is a typical caterpillar with the mouth-parts well developed. These are shown in a ventral view in figure 24. The antennae are minute, and there are only five ocelli (oc) present on each side. They are arranged

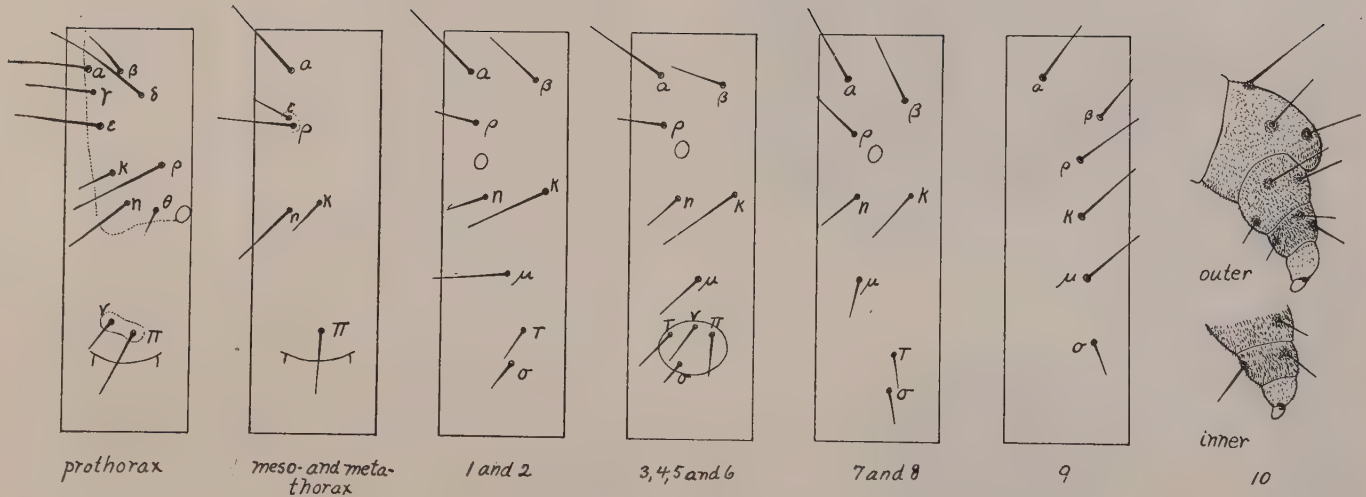

FIG. 25. Setal pattern of 5th instar larva.

in a curved row whose concavity is ventral. The labium is drawn out into a spinneret through which the duct of the silk glands reaches the exterior. The maxillae bear on the inner surface a pair of curved chitinous hooks. Otherwise the mouth-parts are not unusual. On the dorsal surface the adfrontals extend back to the posterior margin of the epicranium and the frons is about one-third the length of the head capsule. The labrum is bilobed and the mandibles bear four "teeth."

The prothoracic legs differ from those of the meso- and metathorax in that the terminal segment bears one claw only, whereas in the case of the latter two pairs of legs the claw is protected by a pair of terminal lappets. The prolegs of the abdominal segments three to six inclusive bear on the planta two rows of crochets, three per row. The anal prolegs have a single crochet only.

The setal pattern of the body of the larva is of some taxonomic importance and is shown in figure 25. In this description the nomenclature of Fracker, though cumbersome, has been followed. The diagrams are made so that the anterior margin of each segment is to the left and the dorsal midline is at the top. Each diagram is that of the left half of each segment projected on a flat surface. Certain segments are alike, and these have been represented by one diagram. The setae of the anal segment do not conform to those of any of the others.

D. Pupa (Text figures 26 to 29)

The pupa is spindle-shaped, about three millimeters long and brown in color. Many of the adult structures are evident, and the head thorax and abdomen are distinct. In the description here given the parts, although often incompletely developed, are named in accordance with the corresponding parts of the adult.

The vertex (vert) occupies most of the dorsal side of the head and is separated from the frons by the Y-shaped epicranial suture, the frons meeting the arms of the suture. The stem of the Y is indistinct. The frons (fr) extends caudally along the ventral side of the head from the epicranial suture to merge into the clypeal region, there being no demarcation between the two. The frons in figure 26 can be easily distinguished by the presence of the pointed cutting plate in its anterior part. This is the so-called "cocoon-breaker" with the aid of which the pupa emerges from the cocoon. The bases of the antennae (ant) are visible on the dorsal side of the head lateral to the epicranium. On the ventral side of the head and lateral to the frons are the eye-pieces (e). The clypeus bears the bilobed labrum, and on each side of the labrum is a small triangular mandibular sclerite. Neither labrum nor mandibles are found in the adult. The labrum bears a pair

of laterally placed setae. The maxillae are prominent and form the pair of medially placed appendages extending caudally from the labrum. Neither maxillary nor labial palpi are visible. The antennae extend caudally almost to the tips of the wings. Be-

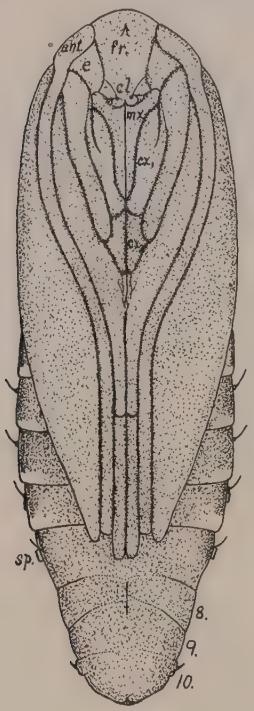

FIG. 26. Female pupa, ventral aspect, much enlarged. *ant,* antenna; *cl,* clypeus; *cx,* coxa; *e,* eye; *fr,* frons; *max,* maxilla; *sp,* spiracle.

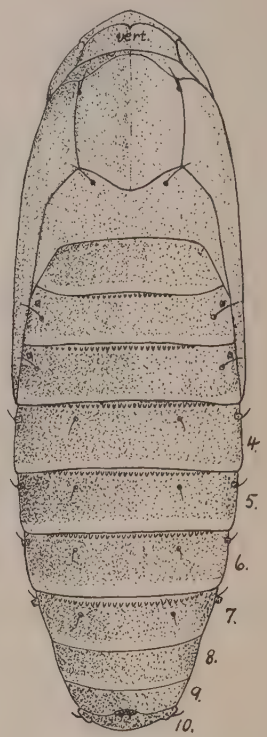

FIG. 27. Pupa, dorsal aspect, much enlarged.

tween the antennae and maxillae lie the folded prothoracic and mesothoracic legs. The tarsal regions of the metathoracic legs are visible between the tips of the antennae, most of this pair of appendages being covered by those preceding. A small part of the metathoracic coxae is visible in the midline posterior to the mesothoracic coxae. The metathoracic legs extend slightly beyond the tips of the wings. The fore wings extend to the seventh segment of the abdomen on the ventral side and conceal the hind wings. The appendages are loosely attached to each other

and are free from the body wall. They overlap, more or less, and the covered parts are quite membranous. When dissected out, the regions of the coxa, femur, tibia and tarsus are visible, although often not distinctly demarcated, and the tibial spurs are prominent. The two folds in the legs occur between coxa and femur and between femur and tibia. The tibia merges into the tarsus.

FIG. 28. Tip of abdomen of male pupa, ventral aspect.

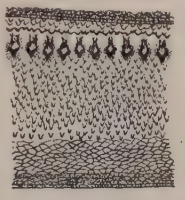

FIG. 29. Section of dorsum of abdomen of pupa.

The female has evidence of two genital openings on the ventral side, one on the posterior border of the eighth abdominal segment and one on the interior border of the ninth. These are short slits in the integument. The male has evidence of one genital opening only (figure 28), on the ninth segment. This condition in each sex corresponds to that of the adult. On the tip of the tenth abdominal segment is the indentation marking the anal opening, and on the lateral sides of this segment are a pair of short strong spines.

On the dorsal side of the pupa the prothorax is constricted in the middle and widens laterally. It lies between the epicranium and mesothorax and abuts on the antennae. The epicranial suture extends to the anterior margin of the prothorax. The mesothorax is a large quadrate sclerite separated by distinct sutures from the prothorax, metathorax, and fore wings. Along its midline it is raised into a very slight ridge. At the anterior-lateral angles are a pair of setae. The mesothoracic wings extend around the body to the ventral side. The metathorax is not so long as the mesothorax and merges indistinctly into the wings laterally. It bears also a pair of setae at the anterior-lateral angles, but these are more approximated than those of the mesothorax. The metathoracic wings are almost entirely concealed by those of the mesothorax, the bases only being visible. There are visible dorsally ten abdominal segments, of which numbers two to seven inclusive bear a pair of setae at the anterior-lateral angles, and numbers one to seven bear laterally placed spiracles. Segments four to seven inclusive bear also a pair of medially placed setae. The spiracles on the first abdominal segment are concealed by the hind

wings. At the anterior margin of the tenth segment is a dorsal tubercle bearing a pair of spines. This and the lateral spines on the tenth segment are purely pupal structures. The dorsal surface of the abdominal segments is covered with minute spines (figure 29) and on segments two to seven inclusive there is a row of heavy spines along the anterior margin.

In the male abdominal segments three to seven are movable, and in the female segments three to six are movable. The terminal segments are immovably united in both sexes.

VI. Life History and Habits

All the data here given, except for field records and other cases specifically mentioned, were obtained from records of individual insects reared in New Haven, Conn., on the gray birch, *Betula populifolia*. The field observations in Connecticut and Massachusetts are also of insects occurring on *Betula populifolia* unless otherwise stated. The period during which records were made covers the years 1924, 1925, and 1926.

The first adults appear the last of June in the region about New Haven, and the last disappear the last part of July. In 1924 adults were fairly numerous July 7th, and the last were seen July 31st. During 1926 adults were systematically collected with a net and by hand during July in one locality, a group of birches just north of Mt. Carmel, near New Haven, and these collections indicated a maximum number of adults were present the fourth week in July. During 1926 the season was later than usual. This species was abundant during the second and third weeks in July, but from the 27th to the 31st it declined in numbers from about a maximum to disappearance.

Pupae were kept at normal temperatures in an out-door screened insectary during 1924, 1925, and 1926. Those of 1924 were collected in the field during the spring of that year; those of 1925 and 1926 were reared in the out-door insectary. Records were kept of the emergence of 104 adults in 1924, 135 in 1925, and 36 in 1926. In 1924 the period of emergence was between June 4th and July 9th (only three emerged before June 23d); in 1925 between June 15th and July 19th, and in 1926 between July 2d and July 21st. The period of maximum emergence during 1924 was between June 25th and July 9th; in 1925 between June 18th and July 10th, and in 1926 between July 2d and July 9th. In 1925 all records obtained after July 1st were of individuals taken from New Haven to Woods Hole, Mass. The early appearance of adults in 1925 may possibly have been due to high temperatures early in June of that year, for, during the first ten days of June, 1925, the mean hourly temperature was 75.2°F. as opposed to 60.6°F. in 1926, and 61.2°F. in 1924 (U. S. Weather Bureau, New Haven, Conn., statistics).

Hutchings (1926) reports that in Ontario the adults are found during July (up to the 25th) with a maximum emergence from July 6th to July 14th. In New Brunswick, Gorham (1922) reports adults abundant the first two weeks of July. It would seem from these reports that the adult insect appears at approximately the same time of year over much of its entire range, being at a maximum during the first part of July.

FIG. 30. Cast pupal skin and cocoon.

From the pupae collected in the field in the spring of 1926 there were secured 54 adults of which the sexes were determined. Thirty-three were females and 21 were males. Of 36 adults secured from laboratory-reared larvae, 19 were females and 17 were males. Although the females outnumber the males, the difference is not sufficiently great to warrant an assumption that there is not approximately an equal number of each sex under natural conditions.

When ready to emerge from the cocoon the insect is in the pre-imaginal stage, and the scales of the imago are easily seen through the pupal skin. The pre-imago works its way forward, probably with the assistance of the spines on the tenth abdominal segment, and breaks through the anterior end of the cocoon. The cutting plate on the vertex is of material aid in the process. When about three-fourths of the length is exposed, the body is held at an angle of about 40° from the cocoon. The pupal skin splits at the junction of the vertex and the prothorax and also longitudinally through the prothorax and mesothorax. The eye-pieces remain attached to the antennae and mouth parts. Figure 30 shows a cocoon and an empty pupal skin in the characteristic position.

According to Snodgrass (1922), the emergence from the cocoon is greatly assisted in *Bucculatrix pomifoliella* Clemens by the presence of three "valves" in the anterior end of the cocoon, these acting as an inclined plane to lift the insect. In *B. canadensisella* these structures are not present. The inner part of the cocoon is woven so that its lateral walls curve in at the base, making a "round corner," and this might conceivably give the pre-imago an upward lift. However, if a pupa is pushed gently forward by hand, it does not emerge at the normal place, but lower down near the base of the cocoon. By simply rolling over in its attempt to extricate itself, the pre-imago would bring the cutting plate in a more dorsal position with respect to the cocoon and thus insure emergence at the normal location.

During the daylight hours the adults rest quietly on the surfaces of the lower leaves of birches, or of the leaves of undergrowth under the birches, or on the lower part of the trunks of the trees. They fly very little unless disturbed, and usually remain motionless for long periods of time. The photograph on plate XVII is a three-minute exposure of a live adult which was carried to and from the camera in the position seen. It shows the adult in the characteristic position. They can be collected very easily by simply inverting an empty glass vial over the quiet insect and then gently tapping the opposite side of the leaf on which it rests. It will quickly fly or run up into the vial. I have collected one hundred in less than an hour from fern fronds in this manner. When disturbed, they fly quickly a few feet, very rarely over five or six, and usually much less. If confined in a bottle they run excitedly for a few minutes when disturbed.

From field observations it seems that as a rule the moths remain near the ground during the day and go up into the trees at about dusk. I have often failed to get them in a net by sweeping birches over my head, when many were secured by sweeping within four feet of the ground. Yet, in the same location, by sweeping the birches at dusk, several were netted very quickly at a height of about nine feet from the ground. In 1926 in one particular locality, moths were very abundant during the day on the fronds of ferns growing under the birch trees, but none were seen on the leaves of the birches which were not lower than four feet from the ground. An investigation of the birches at night with the aid of an acetylene lamp showed that the moths were all up on the birch leaves, and none were on the ferns below. The next day over one hundred were easily caught on the ferns, but none were on the leaves of the trees. At times, however, I have found moths on the leaves of the trees during the day five feet from the ground. Gorham (1922) reports that in New Brunswick he found moths on the birches in large numbers at all hours of the day, but he gives no further information on the distribution.

The moths prefer situations out of direct sunlight, and this may account for their position during daylight hours. This nightly migration into the trees may be affected somewhat by conditions of light, moisture, and amount of undergrowth, but it apparently occurs to some extent wherever conditions are normal.

The response of these moths to light is not very definite, due to their habit of remaining quiescent in one spot unless greatly disturbed. Attempts to make them show either a negative or positive response to daylight in the laboratory gave inconclusive results. Several attempts to attract them to a lighted lantern, an acetylene lamp, and automobile headlights at night in the field failed completely, although they were present in considerable numbers on the surrounding trees and were relatively more active than during daylight.

From the above observations it is assumed that oviposition occurs at night. In the insectary eggs were secured from three to seven days after the adults emerged, but in view of the fact that the moths are very inconsistent about ovipositing in captivity, these data may have to be extended under normal conditions.

It was necessary to place several males and females in a cage in order to ensure a supply of eggs. Although I have no data on the number of eggs laid by any one female, an examination of the oviducts shows that there may be a considerable number, for sixty-two fully formed eggs were dissected out from one female caught in the field, and there may have been several laid before she was captured. This number of eggs was never secured from any female in captivity. From four females thirty-four eggs were secured in one day in a cage, and from these same four, fourteen eggs the following day. They died without further oviposition. These females were reared and laid only the eggs recorded.

The adults have been kept alive in cages out of doors for twelve days after emergence from the cocoon, but they usually die sooner. They have never been seen to feed, and apparently they did not touch a honey-and-water mixture placed in the cage. The presence of a 25 per cent solution of honey in the cage did not prolong the duration of adult life. Certainly food is not a requisite and is not necessary to oviposition. All the adults collected in the field died in a few days, so twelve days probably is a fairly long period of life. Humidity and temperature have considerable effect on this, and moths caught in the field can be kept alive four to nine days if held at 10°-12°C., whereas they die in one to three days at room temperature. They will remain active after an exposure to 7°C. for twelve hours, but when the air is cooled to 5°C. they very quickly become inactive.

The eggs are laid singly on either side of the leaf and on any part of the surface. There is some preference shown for a position beside the midrib or some other prominent vein of the leaf,

but not to the exclusion of the rest of the leaf (plate XVII). Eggs are laid on leaves on all parts of the gray birch. Insectary records for 1924 showed a period of oviposition lasting from July 5th to July 21st, and field observations the same year showed unhatched eggs up to August 7th. No field observations were made during 1925, but gray birches sent from Boston, Mass., to Woods Holt, Mass., on July 9th, carried many unhatched eggs on the leaves. In 1926 eggs were found in the field between July 23d and August 3d, but in view of the fact that they were numerous July 23d and that some of those collected that day hatched July 30th, oviposition must have begun as early as July 16th. Many eggs collected August 3d hatched August 15th, so oviposition occurred as late as August 1st. Oviposition takes place usually during the month of July, and unhatched eggs may be found up to the middle of August. In 1926 the incubation periods of 48 eggs from laboratory-reared adults were 15 days on the average, the maximum being 17 days, the minimum 13 days, and the majority (27) taking 14 days. In all but three cases these eggs went through the incubation period between June 25th and July 13th, a month earlier than normal. They were from laboratory-reared adults which emerged earlier than normal. The other three eggs were incubated at the normal time, between July 31st and August 14th, and they took 14 days each, so the figure for all 48 was normal. This agrees with the period given by Hutchings in Ontario in 1925. The eggs have a high degree of fertility, and those that do not hatch are rare.

A short time before the larva leaves the egg it can be seen curled inside (figure 31). When it emerges, the young larva bores through the bottom of the egg into the leaf, and as it feeds it leaves the egg filled with dark excrement. This habit makes it very easy to determine whether or not the eggs are hatched, for after the larva has left, the egg appears brown or black in contrast to its former translucent condition. For several days the young larva mines close to the egg, but it finally straightens its path and mines in a more or less definite direction.

The larva completes the first and second instars and most of the third in the mine. I have found a head capsule in a mine only twice, but the measurements of the width of the head capsules (p. 444) and the descriptions of the larvae clearly indicate three mining instars. While in the mine the larva is always oriented dorso-ventrally with the leaf; that is, its dorsal side is always toward the upper surface of the leaf. For the first week the mine is extended very little and is always close to the egg, giving a blotch appearance, due to continuous turning of the mine in a small area. A mine six days old measured only 1.5 mm. across the mined area. This larva never makes a real blotch mine, but its excavations are always linear and winding, with slightly

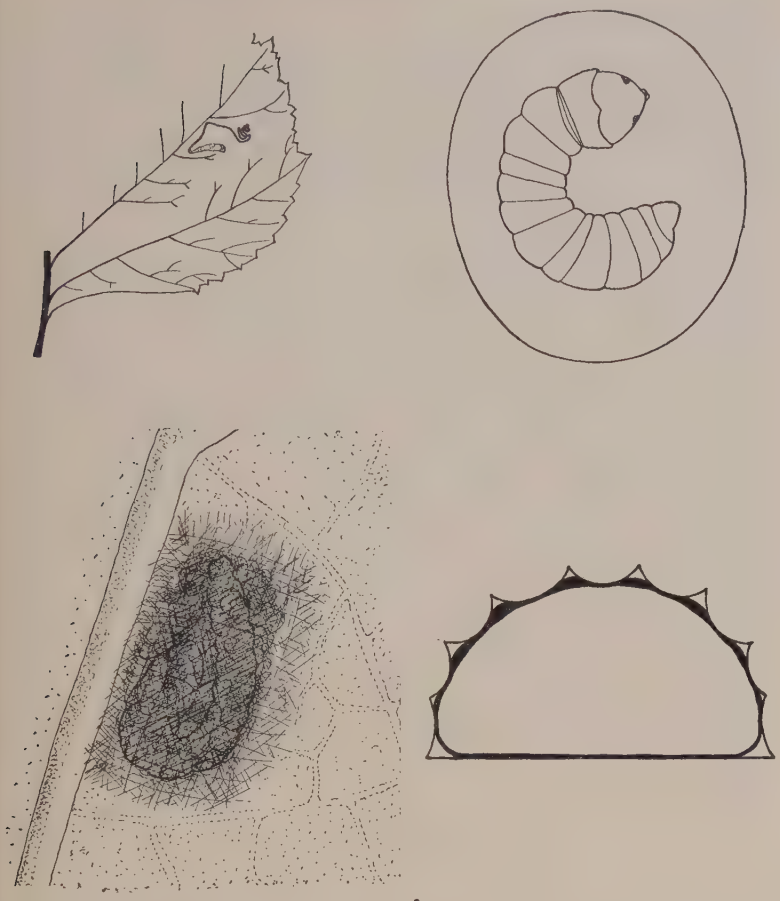

FIG. 31. Larval mine in birch leaf, slightly less than normal size (upper left); embryo in egg (upper right); larva in molting web (lower left); diagram of cross section of cocoon (lower right).

enlarged ends. During the last part of its mining life the larva lengthens the mine very rapidly and broadens it somewhat. Most of the mines are about three-fourths of an inch long when finished (figure 31).

When ready to emerge from the mine, the larva cuts a crescentic opening in the lower epidermis, an operation taking about fifteen minutes. It then works its way out until, by bending its body ventrally, it can grip the leaf surface with its thoracic feet. It then quickly pulls itself out of the mine, the entire performance consuming about two and one-fourth minutes. While emerging

from the mine. the larva spins a few long threads of silk in the shape of a figure "8." I was unable to detect any use to which this peculiar habit could be put. The opening through which the larva emerges is always crescentic, always of the same approximate size, and always cut in the lower surface of the leaf regardless of which surface bears the egg. Hundreds of mines have been examined, and these were made by larvae which hatched from eggs laid on either side of the leaf, and only once an exit was found on the upper surface.

It was thought that both light and gravity might cause the larva to orient itself dorso-ventrally with the leaf, and if this were true, the larva might be made to change its orientation by either inverting the leaf or changing the incidence of light on it. The upper surface of a leaf containing six mining larvae was covered over with black paper, and a mirror was so placed that daylight was reflected on the lower surface. This caused light to fall on the ventral surface of the larvae instead of on the dorsal, as under normal conditions. After an interval of 15 days all the larvae had emerged from the lower surface of the leaf. The change in incidence of light had no effect. A branch of a gray birch bearing several leaves which contained mining larvae was inverted so that the morphologically lower surface was uppermost. This changed the relation of the larvae to gravity. Under natural conditions the leaves never hang horizontally, so the orientation of the larvae is only relative at best. The leaves were examined after an interval of 18 days. Approximately 50 larvae had emerged from the mines, and of these only two had emerged through the morphologically upper, now lower, surface. In view of the fact that occasionally a larva emerges from the upper surface normally, the emergence of these two is of no significance. It is quite evident that changing the orientation to either light or gravity has no effect on the position of the insect relative to the morphologically upper and lower surfaces of the leaf after the mine is well under way; nor will the surface of the leaf through which the larva emerges from the mine be changed by any such procedure. In the above cases all the mines were about half finished when the conditions were changed. The orientation of the larva must be determined after it bores through the epidermis of the leaf from the egg and before it mines to any great extent, for all larvae examined were found with the ventral side toward the lower side of the leaf even when the mine was only two or three days old.

Where the insect is abundant it is by no means unusual to find 25 to 40 mines in one leaf. The mine shows more clearly through the upper epidermis of the leaf than through the lower, but this may be due to differences in the structure of these parts of the leaf rather than to the nature of the mine. The larvae are disin-

clined to gnaw through large veins, and usually the mine turns aside at these obstructions.

The duration of the mining stage varies greatly. The maximum period observed was 50 days, the minimum 13 days. This variation may be due partly to the conditions under which the larvae were reared. In 1924 and 1925 all the larvae were reared at the normal season and fed on leaves in normal condition. During these two years the maximum duration of larval life was 37 days, and the minimum 24 days. In 1926 several larvae were reared from eggs laid by laboratory-reared adults a month earlier than normal—in June, in fact—and the foliage of the birches was in a more rapidly growing and tender condition, especially since these birches were more or less sheltered and were well fertilized and watered. The water content of the leaves must have been greater than larvae would normally meet. All of these larvae but two were in the mines over 32 days, and the average period of 20 was 44 days. Of the two remaining, one was in the mine 16 days and the other 13 days. These 22 individuals are not included in computing the mining period. All of the other larvae reared during 1926, 35 in number, on which records were kept of the mining period, were in the mines 30 days or less. The duration of the mining stage during 1924, 1925, and 1926 was on the average 22 to 27 days for 50 individuals, not including the 22 mentioned above. This figure is probably correct for normal conditions. Hutchings (1926) gives the mining period as seven to eight days in Ontario, but such a short mining period would bring the larvae to a fully grown condition much earlier than they really appear in the field. There is a difference in food plants to be considered, for larvae in New Haven were reared on gray birch, whereas the common birches in Ontario attacked by this insect are the yellow (*B. lutea*) and white (*B. papyrifera*). Nevertheless in Connecticut the larvae appear feeding externally on gray and white birches at the same time, which indicates a similar mining period.

Mining larvae have been observed in the woods about New Haven as early as August 6th, and eggs collected on leaves have hatched July 30th when brought into the laboratory a week earlier. Larvae have been found out of the mines August 6th, but this is unusually early. The period when mining larvae occur around New Haven lies approximately between August 1st and September 15th, with a maximum number present the fourth week in August. The rearing records coincide with these limits.

When once free from the mine, the larva wanders over the leaf for a short time, an hour or two, and then spins its first molting web. A larva has never been seen to feed between the emergence from the mine and the spinning of the web. There may be a difference in different species of the genus, for Chambers (1882)

observed that *Bucculatrix ambrosiaefoliella* feeds two days between emerging from the mine and molting. The larva which emerges from the mine is structurally more like the following external feeding instar than the preceding mining instar. This may argue for possible external feeding, but there is no evidence that it occurs at this time. The exact interval of time between emergence from the mine and either beginning or completion of the first molting web was determined for three larvae, all typical cases. One larva emerged from the mine at 8.20 A. M. and completed its web at 10.45 A. M.; one emerged from the mine at 9.40 A. M., began its web at 10.40 A. M., and completed it at 12.10 P. M.; one emerged from the mine at 10.09 A. M., began its web about 10.42 A. M., and finished it at 12.12 P. M. The larva often selects a position beside a large vein for its web, but it will also spin on the flat upper surface of the leaf. There seems to be a preference for a hollow over which the "roof" of the web may be spun, as the angle between the base and sides of a glass bottle or the hollow beside the midrib of the leaf. Having selected a suitable location, the larva lays down a thin basal "floor" web on the surface of the leaf. This is about 1.5 mm. in diameter. Then it spins another web over this, making long tacks from side to side by swinging the entire thorax and the first two abdominal segments from one side to the other. The body is held facing out and the threads are always straight. In shifting its position the larva swings the abdomen quickly almost 180 degrees. The periphery of the web is thus built up first, the center being weak. A series of short tacks is now made over the "frame" of long threads, and the center is strengthened. This is followed by a series of short tacks all around the edge, a proceeding which evidently strengthens the web. A hole is quickly made through the web near the center, and the larva crawls in head first between the "floor" and the "roof." In crawling into its molting chamber the larva doubles ventrally so that its back is down on the "floor" and its feet touch the "roof"; that is, it is oriented dorsally to the leaf. Before it is all inside, the larva swings its head to and fro, weaving a mat on the under side of the "roof." Since the diameter of the web is not much more than half the length of the larva, the latter is forced to turn around, and when completely inside, its head almost touches the last abdominal segment, the body being bent in a U shape and to the right or left (figure 31). It is plainly visible through the web. The larva is not content with getting inside, but actually makes a turn around its molting chamber. All this time it swings its head, weaving figure "8" loops, and in due time it incidentally has to cover the hole in the "roof" by which it entered. Although this opening is always covered, the larva seems to make no deliberate attempt to cover it, doing so eventually as it works around inside. Most of the weav-

ing is done after the larva is inside. In two instances to which particular attention was given, the time spent weaving prior to entering the web was eight minutes in each case, and the weaving time inside the web was 56 and 52 minutes respectively. In two other instances the larvae were weaving inside the web 60 and 70 minutes respectively. The principal part of the web is woven from the inside and is supported on the lighter structure previously woven from the outside. The entire process of spinning the web takes about one or one and one-half hours, varying somewhat with the larva. The procedure is essentially as described by Snodgrass for *Bucculatrix pomifoliella*. When the larvae are numerous, the birch leaves in August and September are spotted on both sides with many white webs. I call these "molting webs" rather than "cocoons," "pseudococoons," or "cocoonets," as termed by others, because I believe the word "cocoon" should be restricted to that structure, in which the pupal stage is passed.

Having completed its web, the larva retracts its appendages somewhat and remains quiescent a day or two. The tarsal claws and the crochets of the prolegs are not attached to the web, the larva lying freely with its ventral side away from the leaf. If the upper part of the web is removed, the larva falls out. Under such conditions it must molt in some sort of a chamber or fall off the leaf, which might be disastrous, for if food is not available after the molt, the larva dies in a few hours. The small size of the web holds the insect tightly, and the strong attachment to the leaf secures the web against being washed off or lightly brushed off. The web also offers protection from such enemies as ants during a period of helplessness. It is not essential to the process of molting, and seems to be an obstacle to quick molting rather than an aid. If removed from the web, the larva molts perfectly normally. Inside the web it has to pull itself around to get clear of its old skin. After one or two days in the web, the larva molts, and in a few hours, sometimes in one hour, it breaks out through the edge, at the junction of "roof" and "floor" (plate XVII). In molting, the head capsule separates from the rest of the old skin and is cast off first anteriorly. The larva then works its way clear of the remaining skin, casting it off the posterior segment of the abdomen. The molted head capsule and skin are left inside the web and separate from each other.

The manner of leaving the web shows how precisely instincts can regulate action. After it has molted, the larva normally bites a hole through the side of the web and emerges, but before molting it will not bite through and hence cannot get out even though it so desires. An individual which had just entered the web (in this case the second molting web) was rendered inert by hydrocyanic acid gas. After four minutes it regained sensibility and for the next 14 minutes made spasmodic movements while recov-

ering, being apparently normal at the end of this time. It then attempted to get out of the web by pushing against the sides, having "forgotten" the reason for its imprisonment. It pushed vigorously back and forth for seven minutes, stretching the sides of the web in its endeavors to escape, but to no avail. Half a minute's work with its mandibles would have set it free, and had it molted, escape would have normally been accomplished in this manner. The instinct to bite its way out was totally lacking. Finally it began to move around inside the web and spin irregularly, then it began to weave the normal figure "8" loops, and in 28 minutes the web was finished. The larva cannot use its only means of escape from the web until the act of molting is accomplished. This individual later molted and developed normally, not being in any way injured by its treatment.

If removed from the web before it is finished, or, if it is finished, before the pre-molting quiescent stage begins, the larva will spin another web or as much of another web as is possible and will molt normally. An effort is always made to complete another web, but sometimes lack of the necessary silk, or exhaustion, or some other factor, compels the larva to stop after a few strands have been spun, and it then molts in the most convenient place. If it has entered on the quiescent stage prior to molting and has become fixed in the shape of a horseshoe, it does not straighten out when taken from the web, but retains its curved shape until it molts.

Because of the fact that the larva leaves its web so soon after molting, the duration of the instars has been calculated to include the time spent in the web made by the particular instar in question. Thus the feeding period plus the subsequent quiescent period spent in the web gives the length of the instar.

The time spent in the first molting web is much affected by temperature, and usually varies between one and four days in this climate. Many larvae spend less than 24 hours in this web, but most of the larvae are in it about two days. If this period is added to the days of mining life, we get a period of 24 to 29 days for the first three larval instars. This is not remarkably long when compared to the length of the next two instars, which together total about two weeks.

After emerging from its first molting web, the larva feeds from one to nine days, the individuals varying greatly under the same conditions. If food is withheld from the newly molted larva, it dies in a few hours, a much shorter time than if starved after feeding a day or two. This is probably the result of remaining a day or two in the molting web without feeding. During the fourth instar the larvae are restless and wander about more or less. This probably accounts in part for the variation in the length of the instar, for the rapidity of development is much

dependent on the amount of food eaten. The average duration of the feeding period for 73 individuals recorded was about four days. In only one instance was the feeding period as short as one day. Temperature affects the duration of this period to some extent, as will be brought out later. The effect of different species of birch as food will also be discussed in another section of this paper.

The feeding occurs normally on the lower side of the leaf, and the veins and the upper epidermis are left intact. The entire leaf is never consumed. It is due to this habit of skeletonizing a leaf that the insect bears its common name. The larvae will eat whichever surface of the leaf is toward the ground, and normally this is the lower epidermis. A birch leaf was inverted so that the normal lower surface was uppermost and covered with a black paper. A mirror was so placed that it reflected light on the leaf from below. The larvae normally feed on the lower side of the leaf, and under normal conditions this side is not so light as the upper. If the larvae fed on the lower side of this inverted leaf, they would feed on the lighter side and at the same time on the side normally uppermost. The two sides of the leaf differ in physical as well as chemical constitution of the surface. Of ten larvae placed on the upper side of this inverted leaf, four migrated to other parts of the plant (a normal movement), one remained on the upper side and was feeding when examined, and five went to the lower side of the leaf and were feeding. Seventeen hours elapsed between the placing of the larvae on the leaf and the final observation. Larvae were then placed on the uppermost side of an inverted leaf and watched. Usually they wandered about restlessly for a time until they came to the edge of the leaf. They then turned to the side underneath. Light reflected on the lower surface by a mirror seemed to have no effect. At times movement to the lower surface was long delayed and at times direct. It very evidently is a reaction to gravity that impels these larvae to feed on the lower leaf surface and not any dislike for the upper surface nor any negative reaction to bright light. What factors developed the habit of feeding on the lower surface only is another matter. The habit of the larva is to feed continuously over a limited area, and it does not wander far unless the food supply gives out. If disturbed, the larva usually drops off the leaf, spinning a long thread as it falls. After falling a few inches it hangs on the end of the thread a moment and then quickly ascends. The thread is spun out the tip of the spinneret, and when the larva stops its descent, it is attached to the end of the thread by means of the spinneret. When it ascends the thread, it moves its head rapidly back and forth and winds the silk on the prothoracic legs which are held forward. If there is too much silk for the prothoracic legs, the mesothoracic legs are brought into use. On

regaining its support, the larva simply drops the bundle of thread and walks away. This performance can be easily watched under the binocular if a larva of the last instar is used. The spinning activities of the larva, the quickness with which it drops from a leaf, and the distance it drops are much greater in the last instar than in the fourth. The speed with which these little insects can spin a thread while falling a few feet is remarkable. If touched, they snap the body back and forth rapidly and thus wriggle off the leaf and drop toward the ground. Yet after they have fallen some distance, they suddenly check their descent and can be seen to be hanging by the end of a thread. The silk of which this thread is formed must be spun from the silk glands and out of the spinneret as rapidly as the larva falls. The act of spinning apparently occurs automatically when the larva is disturbed.

Because of their small size and their greenish color, together with the comparatively small amount of leaf tissue eaten, larvae of the fourth instar are not so noticeable as those following. In localities where Bucculatrix is abundant, however, ten to fifteen larvae may often be found on one leaf. Heavily infested birches frequently have 25 larvae of the fourth and fifth instars feeding on each leaf. During the majority of seasons no such number is likely to be present.

The fourth instar molts as did the third, in a white silken web. This web is larger than the previous one, being about 2.5 mm. across. The larva builds the web and lies in it as previously described, being clearly visible. There is a slight difference in structure, as this larva weaves an elliptical mat after it is inside the web. This thickened part gives the second molting web a characteristic appearance, as the first molting web has this structure to only a very slight degree. The time spent in this web varies normally from one to three days, the 75 individuals recorded averaging about two days. This is, of course, affected by the temperature, as was mentioned before. When added to the feeding period this figure gives the length of the fourth instar as about six days.

The larva molts as before and emerges from the second molting web as from the first. It normally feeds on the under side of the leaf, skeletonizing it (plate XVIII), and in this instar the feeding is much more extensive. The injury to the foliage is most noticeable at this time, usually during the last of August and most of September. If the larvae are present in large numbers, all the parenchymatous tissue is consumed, and the leaf dies and drops from the tree. These larvae show greater spinning activity than those of the former instar and may be seen suspended from the leaves in great numbers in seasons of abundance. They feed from two to ten days, the period varying with the individual and being affected by climatic conditions, and an average of 48 recorded individuals gives a period of nearly seven days. This

period includes the time from emergence from the second molting web to the spinning of the cocoon. Toward the last part of the feeding period the gonads are clearly seen through the dorsal skin of the abdomen of the larva. About twelve hours before the time when the larva will begin to spin its cocoon, it stops feeding. This interval of time varies considerably and may be much less. By this time the larva has turned brown in color, due to the color of the large silk glands which run almost the entire length of the body. When ready to spin the cocoon, the larva drops from the place of feeding to the ground, spinning out a long thread as it goes. Larvae may sometimes be seen suspended from a thread about fifteen feet long. If the trees on which they are feeding are shaken, these fully grown larvae drop to the ground quickly and in considerable numbers. Having reached the ground, they crawl under a stone, a fallen branch, a leaf, or any other object lying on the ground and spin their cocoons on the under side of this. Sometimes the cocoon is spun on the ground itself. In captivity they will frequently place the cocoons on the sides of the cage close to the base. I have reared hundreds of larvae, and they all have dropped to the ground or close to it to pupate. Fletcher (1893) mentions finding three cocoons on the twig of a birch, but all the cocoons which I have found in the field have been on fallen leaves or other objects lying on the ground.

The manner in which the larva spins its cocoon is characteristic of the genus and quite unique. The earliest description of this process in the genus *Bucculatrix* is by Lyonet, who wrote to Réaumur, December 22, 1744, concerning the larva of *B. ulmella* and its cocoon. This description was not published until 1832 and has been referred to in the historical part of this paper (page 396). De Geer, in the first volume of his "Mémoires," published in 1752, described the cocoon of *B. frangulella* (see page 395), and Snodgrass in 1922 likewise described the manner in which *B. pomifoliella* Clemens wove its cocoon. These three papers go into the details of the process by which the larva lays down its threads, and from a microscopic examination of the cocoon of *B. canadensisella* it is apparent that this larva weaves its threads in precisely the same manner as does *B. pomifoliella*. The general process of weaving is similar in all four species, differing only in a few details. Chambers (1882) described briefly the formation of the cocoon by *B. ambrosiaefoliella* Chambers, and McGregor (1916) gave a brief description of the finished cocoon of *B. thurberiella* Busck. In 1892 Fletcher briefly described the general procedure of weaving by *B. canadensisella* Chambers, and in 1893 Lintner mentioned the same subject, but the latter's description is not correct, and Fletcher's description is not detailed.

The larva of *B. canadensisella* Chambers first lays down an oval mat to serve as a base for its cocoon. It does not previously weave a palisade of poles around the site selected, as do many

species of the genus. It then commences at one end of the mat to weave an outer supporting ridged structure of comparatively coarse threads (about .005 mm. thick), facing the work and backing away as the woven structure progresses over the mat in an arch. The ridges are formed by the ends of a series of loops made from one side to the other. The diagram in figure 32 gives

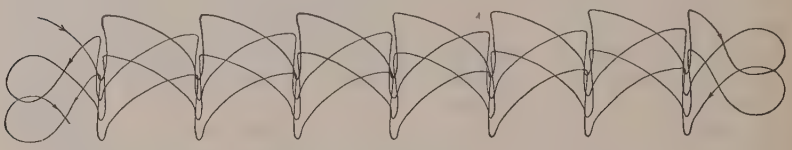

FIG. 32. Diagram of method by which larva of *Bucculatrix pomifoliella* Clemens weaves its cocoon. After Snodgrass.

the principle. Between ridges the threads cross diagonally. This figure is from Snodgrass (1922) and gives his conception of the actual motions made in weaving. As the cocoon becomes higher, the larva raises the anterior part of its body, and the radius of the structural arch is gauged by the raised part of the body as it swings from side to side, most of the body being fixed in the midline of the oval base. Possibly the prothoracic legs are used in the weaving to aid in guiding the work, as mentioned by Snodgrass and Lyonet. Certainly these legs are held up to the structure. When the cocoon is about two-thirds finished, the larva enters it, turns about, and crawls out until its head reaches the other end of the mat. It now has its anterior end outside of the cocoon but its posterior end in the cocoon. Beginning to weave exactly as before, the larva builds up the last third of the cocoon to meet the previously formed two-thirds, gradually enclosing itself as it works. When the two sections meet, they are joined by cross threads. The architecture is not perfect, for the ridges of the two sections rarely coincide, and sometimes the heights of the sections are not equal. The result is a break in the continuity of the ridges at the junction and often a sag in the contour of the cocoon. A completed cocoon is shown on plate XVIII. This outer structure is not closely woven and the insect can be seen clearly inside. It is, however, stiff and gives support to the lining which is to be woven. The sides meet the oval base perpendicularly.

Having completed its superstructure, the larva weaves a closely knit lining of fine threads (about half the thickness of the threads of the supporting structure) all around the inside by swinging its head in figure "8" loops. Where the walls of the superstructure join the base, the cocoon does not follow but makes a round

corner, as the diagram in figure 31 shows. It is this lining which makes the cocoon opaque. Snodgrass has described a series of "valves" in the anterior end of the cocoon of *B. pomifoliella* Clemens, but in the cocoon of *B. canadensisella* these are not present. The cocoon when first finished is almost pure white, but it soons turns brown. This brown color is due not to the pupa inside, for it is present before the prepupa molts, but to a change in the color of the silk when exposed to air. The time necessary to complete a cocoon is from eight to sixteen hours normally. Inside the cocoon the larva remains two or three days before pupating. This prepupal period plus the feeding period makes the fifth instar about nine days long on the average. The larva molts in the cocoon in a manner differing slightly from that which takes place in the molting webs. In the webs the head capsule is cast off entire and anteriorly while the rest of the larval skin is worked posteriorly off the anal segment. In the cocoon the entire larval skin, head capsule included, is worked off posteriorly.

The individuals which were reared in the outdoor insectary under normal temperatures in 1924 pupated from September 4th to September 25th; in 1925 from September 8th to September 13th; in 1926 from September 11th to September 23d. This does not indicate the time of disappearance of the last larvae in the field. During these three years an examination of birches about New Haven was made in order to determine the normal close of the larval period. In 1924 the last larvae were found October 9th; in 1925, September 19th; and in 1926, October 9th. The early disappearance of larvae in 1925, although not caused by any apparent natural enemy or unusual climatic condition, was exceptional. It may have been caused in part by an early season starting the life cycle earlier. In view of the fact that larvae will feed at 48° to 50°F. and will eat birch leaves until they begin to turn yellow. very few are caught before pupation by cold weather or lack of food.

The total larval life occupies from 38 to 46 days, as a rule, as the table on page 441 indicates. This is not an average of the completed larval life of a number of insects, but an average of the separate stages of many individuals, rather few of which completed the entire larval period while under observation. Nine larvae carried through from egg to pupa in 1926 averaged 41 days, the maximum being 45 days, and the minimum 36 days. This is as close as could be expected to the 38 days given in the summary for 1926. In the table below, the larval life from the hatching of the egg to the spinning of the cocoon is given for the nine individuals mentioned above. Two days as prepupa should be added to the six days of feeding in the fourth instar to give the total larval period of 41 days. It will be noticed that the larva does not accelerate through one instar if slowed down on a previous

instar, but that any retardation during the growing period is permanent as regards time. This is borne out by the other records. Seven larvae were reared in the laboratory in vials containing moist sand, and were under identical environmental conditions. The figures for the stages are given on pages 474-478 (larvae 131-137). The most slowly growing larva was six days in the fourth instar and was feeding six days and fifteen hours in the fifth, while the most quickly growing larva was four days and nine hours in the fourth instar and was feeding four days and nineteen hours in the fifth. It will also be noticed that the quiescent period spent in the molting web is independent of the length of the feeding period, and as the feeding period grows shorter, the proportion of time spent in the web during one instar grows greater. In larva number 9 in the table below, two-fifths of the fourth instar is quiescent, and in number 1, three-sevenths, but in numbers 2 and 4, only one-fourth of the fourth instar is quiescent. The effect of food and temperature on larval growth will be discussed later.

TABLE 1. COMPLETE LARVAL PERIOD

No.	Hatched	In Mine	In Web 1	Total Days	Feeding	In Web 2	Total Days	Feeding	Spun Cocoon	Total Days
1	8-14-26	26	2	28	4	3	7	5	9-23-26	40
2	8-11-26	22	2	24	6	2	8	7	9-19-26	39
3	8-11-26	22	3	25	4	2	6	6	9-17-26	37
4	8-14-26	20	2	22	6	2	8	6	9-19-26	36
5	8- 7-26	27	2	29	4	2	6	5	9-16-26	40
6	8-11-26	30	1	31	5	3	8	4	9-23-26	43
7	8- 7-26	27	3	30	5	2	7	6	9-19-26	43
8	8-14-26	20	2	22	5	2	7	6	9-18-26	35
9	8- 7-26	22	1	23	3	2	5	6	9-10-26	34
Aver.				26±.74			7±.22	6±.16		39±.7

The chart below (text figure 33) gives the periods during which the various stages may be found in the field around New Haven, Connecticut. These limits are computed from field observations and data obtained in the insectary and are broader than actual field observation alone would give. From what notes there are of the occurrence of this insect elsewhere, it seems likely that these periods are approximately correct for the entire region in which the insect is found.

TABLE 2. SUMMARIZED LIFE HISTORY OF *Bucculatrix canadensisella* CHAMBERS

Year	Adult		Egg		Mining instars					Fourth instar					Fifth instar				
	Number of individuals	Period of emergence	Number of individuals	Days in egg	Number of individuals	Days in mine	Number of individuals	Days in 1st molting web	Total length of instars	Number of individuals	Days feeding	Number of individuals	Days in 2d molting web	Total length of instar	Number of individuals	Days feeding	Days prepupa	Total length of instar	Days of larval life
1924	104				5		44			19		19			16				
max.		June 4				32		4			7		3			8			
min.		to				25		*1			1		1			2			
aver.		July 9				27		2	29		3		2	5		6	†2	†8	42
1925	135				10					34		34			27				
max.		June 15				37					8		3			10			
min.		to				24					2		1			6			
aver.		July 19				29		2	31		3		2	5		8	†2	†10	46
1926	36		48		35		34			20		22			15				
max.		July 2		17		30		3			6		3			7			
min.		to		13		18		1			2		1			4			
aver.		July 21		15		22		2	24		4		2	6		6	†2	†8	38

* Several larvæ were in the first molting web less than 24 hours.

† The length of the prepupal stage was determined by removing several individuals from the cocoon at various times after the cocoon was completed. This stage was determined in 1926 and the 1926 figure used in computing the length of the stages in 1925 and 1924.

VII. Determination of the Number of Instars

It is very difficult to determine the number of instars by examining the mines for head capsules. A large number of mines were examined for this purpose and in two cases one capsule was found. The extent of growth and the morphological changes undergone during the mining period indicated at least two and per-

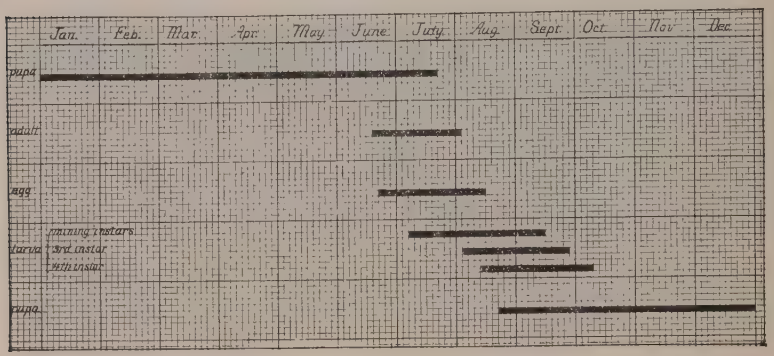

Fig. 33. Seasonal occurrence of the various stages of *B. canadensisella* in the vicinity of New Haven. The larval periods shown should read mining instars, fourth instar and fifth instar.

haps three larval stages. A number of larvae were collected in the field and the width of the heads measured. The head is not subject to growth changes during any one instar, and according to Dyar (1890) a constant numerical ratio exists between the widths of the heads of any two successive instars of a larva. If the heads of two successive instars are measured, or if a large number of miscellaneous heads are measured, the ratio for the species can be determined and the possibility of missing an instar removed. Any dimension of the head may be used, but the width is the most convenient.

Several embryos which had developed to the stage where they were about to emerge from the egg and where no further growth of the head could be expected were measured. These were all mounted in Canada balsam. As seen by the table on page 444, the average width is .078 mm., and nine of the twelve measured .076 mm., which latter figure may be considered normal. It is to be expected that the measurements for the first instar would conform to this figure, and of the sixty-one mining larvae measured, eighteen either equal this figure or closely approximate it. All but two of the eighteen equal it. The average width for the first instar is then .077 mm. Thirty-six of the sixty-one measure .114

mm. in width or very nearly so, thirty-four measuring just that figure and the other two measuring .120 mm. The normal and average for this group is .114 mm. The remainder of the mining larvae measured, thirteen, all give a head width of .171 mm. Two larvae were secured just as they left the mine and before they began to weave the molting web, and their heads measured. Both gave a width of .171 mm. These two are marked (ex) in the third column. This checked the group giving this measurement as the last mining instar. Also four larvae were found in the process of molting and with the head capsules just far enough off to permit the measurement of both the old capsule and the new head. Two of these gave the width of the old capsule as .076 mm. and the new head as .114 mm., while the other two gave .114 mm. and .171 mm. for the two widths. This gives a check on the three groups. According to Dyar's principle we should expect

$$\frac{.114}{.077}=\frac{.171}{.114}=R$$

In this case the ratio "R" is 1.5, and the number of instars in the mine is, as the figures indicate, three. To further check this principle, a number of external feeding larvae, also collected in the field, were measured. I have placed these forty-one larvae in two groups as the table shows. According to the principle used above, the measurements should be ,257 mm. (.171 x 1.5 = .257) and .385 mm. (.257 x 1.5) for the fourth and fifth instars. (The actual number of externally feeding instars was determined by actual observation, of course.) In the fourth instar the average width was found to be .245 mm. for the nineteen individuals, with a variation between .228 mm. and .257 mm. The last instar, containing twenty-two individuals, gave an average width of .353 mm. with a variation extending from .304 mm. to .390 mm. It is questionable whether the two larvae whose head widths are .304 mm. belong to the fourth or the fifth instars if one judges by these two measurements alone. The average width is less than that expected in both the external feeding instars, but even so the measurements are sufficiently closely grouped in each case to determine the instar. It is to be expected that the more nearly the larvae approach the fully grown condition, the more widely will they vary in size, for the absolute extent of variation in size under normal conditions increases with age. The change in environment from the mine to the surface of the leaf, with its difference in manner of feeding involved, would also change the shape of the head, because mining larvae have relatively flatter heads. The actual measurements obtained of the heads of the first three instars is much closer to the ideal than would usually be expected.

TABLE 3. HEAD WIDTHS OF THE LARVAE OF *B. canadensisella* CHAMBERS

(All dimensions in millimeters)

	Embryo	First instar	Second instar	Third instar	Fourth instar	Fifth instar
1	.076°	.076*	.114°	.171° (ex)	.257′	.323′
2	.076°	.076*	.114°	.171° (ex)	.247′	.352′
3	.076°	.076*	.114°	.171°	.247′	.323′
4	.076°	.076*	.120°	.171°	.228′	.304′
5	.086°	.076*	.114°	.171°	.247′	.371′
6	.076°	.076*	.114°	.171*	.238′	.380′
7	.086°	.076*	.114°	.171*	.247′	.380′
8	.082°	.076*	.114°	.171*	.247′	.390′
9	.076°	.076*	.114°	.171*	.228′	.380′
10	.076°	.076*	.114*	.171′	.247′	.371′
11	.076°	.076*	.114*	.171′	.238′	.361′
12	.076°	.076*	.114*	.171′	.247′	.361′
13		.076*	.114*	.171′	.247′	.304′
14		.095*	.114*	.171′	.247′	.370′
15		.076′	.114*	.171′	.252′	.380′
16		.082′	.114*		.228′	.352′
17		.076′	.114*		.250′	.361′
18		.076′	.120*		.257′	.361′
19			.114*		.247′	.352′
20			.114*			.332′
21			.114*			.380′
22			.114*			.370′
23			.114*			
24			.114*			
25			.114*			
26			.114°			
27			.114°			
28			.114°			
29			.114′			
30			.114′			
31			.114′			
32			.114′			
33			.114′			
34			.114′			
35			.114′			
36			.114′			
Theoretical average			.114	.171	.257	.385
Average found	.078±.0008	.077±.0001	.114±0001	.171±0.0	.245±.0013	.353±.0036
Standard deviation	.0039	.0045	.001	.000	.0085	.025
Greatest deviation from theoretical	.008	.008	.006	.000	.017	.081

In previous descriptions of the genus *Bucculatrix* it has been tacitly assumed or explicitly stated that the mining period included one instar only, and that the insect always molted on the surface of the leaf. The only mention I have found of a larva molting in the mine is in a description of the larva of *B. ambrosiaefoliella* Chambers by Chambers (1882) in which he states that the larva in question molts once in the mine, once on the surface of the leaf, and once in the cocoon. It would be well, however, to apply Dyar's principle, at least to the early stages, before making any definite statements regarding other species of this genus.

All the measurements given above were made with an ocular micrometer, using a low power of the microscope. The smallest micrometer scale division was .019 mm., and it was found impracticable to interpolate to less than one-fourth this, a measurement of .005 mm. The embryos measured were all mounted in Canada balsam. The larvae of the first three instars, marked with a small circle, "°," were also mounted in balsam; those marked with an asterisk, "*," were mounted in glycerine; and those marked with an apostrophe, "'," were specimens preserved in alcohol. All the fourth and fifth instar larvae were preserved in alcohol after fixation in Gilson-Carnoy's fluid.

VIII. Food Plants

The plants on which the larvae feed are restricted to the genus *Betula,* with the possible exception of the alder, *Alnus incana.* Johannsen, who reports (1911) the single instance of larvae attacking the alder, has also reported (1910) the presence of larvae on red oak. There are other species of *Bucculatrix* which feed on oak, one of which is very common in Connecticut, and it is very probable that the larvae referred to by Johannsen were not *B. canadensisella.* The mines in oak leaves are very similar to those of the birch skeletonizer, but the cocoons are white and are found on the trunk and branches of the trees. I have not bred this species, but Forbes (1923) gives *B. ainsliella* Murtfeldt and *B. pąckardella* Chambers as indigenous to northeastern United States, and both feed on oak. Alder is closely related to birch, and although the larvae of *B. canadensisella* did not survive in laboratory tests, through one complete instar on *Alnus* (*rugosa?*), under different conditions they may possibly feed on this plant. Of the species of birch on which this insect lives, four are native and one imported from Europe. These are *Betula populifolia* (gray birch), *B. papyrifera* (paper or white birch), *B. lutea* (yellow birch), *B. lenta* (black birch), and *B. alba* (European white birch) respectively. The European birch is a common ornamental tree in northeastern United States and southeastern Canada, and varieties are called the cut-leaf or weeping birch. This tree in Canada seems to be a favorite food plant, but in the vicinity of New Haven it is not quite so severely attacked as the gray birch.

Of the four native food plants, the black birch seems to suffer least, although Maheux reports (1926) that in Quebec this tree has been heavily skeletonized. Which of the other three is most severely injured seems to depend on which is prevalent in the locality. In most of Connecticut the gray birch is the preferred food plant, but on the shores of Highland Lake, where the white and black birches are the only two species common, the white birches were heavily skeletonized in 1925; and in other parts of

Litchfield County, where yellow birch is quite common, it is a favorite host. In Ontario and throughout the Great Lakes regions, the yellow and white birches are the trees which suffer most. The black birch in Connecticut is very slightly injured and usually is untouched, even though its branches intermingle with those of the white and gray birches when these two bear thousands of caterpillars. In laboratory tests the larvae ate the leaves of the black birch very readily. These larvae were taken from gray birch and fed on black birch during the fifth instar. Five of the ten larvae pupated normally, although the duration of the instar was 173 hours on the average as compared with 117 hours for the control. This delay in maturing was partly due to the delay the larvae experienced in getting accustomed to the new food plant.

The red or river birch (*Betula nigra*) is not a common tree in northern United States and southern Canada, and this may be the reason that it is not reported as being attacked by this insect. New England is about its northernmost range, and here it is found only in a few scattered places along river banks. No attempt was made to rear the larvae on the leaves of this tree, as the material is not readily available, and there are no references in the literature to it as a food plant.

There are four other genera of plants belonging to the same family as the birches and growing very commonly in the same localities as these trees. These are *Ostrya* (hop hornbeam), *Carpinus* (ironwood), *Alnus* (alder), and *Corylus* (hazelnut). Under natural conditions I have never observed any of these plants attacked by the larvae of *B. canadensisella,* although they very frequently intermingle with the birches. In the laboratory the larvae have been forced to eat the leaves of *Alnus* but could not maintain themselves on these leaves. The larva itself has really very little to do with the choice of food plants, for this is a leaf-mining insect in the early stages, and if the egg is not laid on a leaf in which the larva can live, death results. Even during the external-feeding stages it is very questionable if a larva could survive long enough to travel from an unfavorable to a favorable plant unless the two plants were very close together.

In an attempt to secure eggs on the leaves of the alder, I placed two alder twigs, each bearing two or three leaves, in a cage with five males and five females. One of the twigs had been dipped in the distillate from an aqueous extract of birch leaves, and the other was normal. The moths were collected in the field. Two females lived six days, one five days, one three days, and one two days, but no eggs were laid. In another similar trial with one female and six males, the female lived four days but laid no eggs. In view of the fact that the females are loath to lay eggs in captivity, the results are merely indicative and not conclusive.

An attempt was made to force larvae to eat the leaves of the alder and the black oak. All these larvae were collected in the field on gray birch. Five larvae in the first molting webs were placed in vials with the leaves of each plant. On the alder all five larvae died in three and one-half days or less without feeding. On the oak some feeding occurred and one larva went through the fourth instar in eight days and then died, starved, in fourteen. Three of the others died of starvation in four and one-half days or less, and one was accidentally killed. Although alder is more closely related to birch than is oak, yet the black oak was preferred as food, though it could not sustain the larvae. Ten larvae were then similarly kept with the leaves of these two plants, but the leaves were previously dipped in a distillate from an aqueous extract of birch leaves. It is sometimes possible to make insect larvae eat materials that have the odor of their food plants. Of the ten larvae used in this case, five were in the fourth instar and five in the fifth. On the alder both instars fed a little. One fifth-instar larva lived ten days, and two fourth-instar larvae lived seven days, but none went through a complete instar. On the oak there was more feeding than on the alder. One fifth-instar larva spun a cocoon after five days, and three others lived between seven and nine and one-half days. One fourth-instar larva lived sixteen and one-half days, molting meanwhile, and three others lived between five and one-half and eight days. In only one instance on the oak was the fourth instar completed. Although the distillate from the birch extract made the alder and oak more attractive to the larvae, and they ate relatively much more of the leaves when so treated, they did not show any growth except in the one instance mentioned above. All but one gradually shrunk in size and finally died of starvation before molting. Control larvae fed on the gray birch were normal in development. On this basis the possibility of larvae under natural conditions living on either oak or alder seems remote, and the reports of feeding on these plants were probably cases of misidentification of the insect in question.

Under laboratory conditions the larvae from the gray birch very readily eat leaves of paper and black birch, and larvae from paper birch just as readily eat leaves of gray birch. In all cases the larvae will mature. The trials conducted were not sufficiently extensive to determine whether or not there is a racial difference in the individuals from different host plants. This racial difference would be primarily manifested by the oviposition response of the adult, and difficulties in securing eggs consistently from females have precluded any definite experimental evidence on this matter to date. When the larvae were reared in the laboratory they were placed on the plants under trial, and if they left these plants, they were put back again. This was continued until they

ate the leaves or died. Under normal circumstances, no such condition would be met, and it is conceivable that the larvae might well starve to death in the midst of food which would sustain life, but which, for various reasons, they would not eat. The preference for birch as food, as concerns the larvae, is partly controlled by a chemical sense, for they eat oak and alder leaves more readily when these are first dipped in a distillate from an extract of birch leaves.

IX. Factors Affecting Abundance

The phenomenon of periodic outbreaks of *Bucculatrix* has been dealt with historically in previous pages. Some of the factors which have a bearing on the abundance and rate of increase of this insect deserve consideration. These may be grouped under food supply, climate, and natural enemies (including diseases). Man has not as yet played any direct rôle in the control of this species.

There is no scarcity of food plants in the northern United States and southern Canada, and the endemic population of Bucculatrix has no apparent effect on the growth of birch trees. Between outbreaks the larvae are scarcely noticeable. Paper birch forms a great part of the subarctic transcontinental forest and is a very common tree as far south as the Great Lakes and central New England. Gray birch is common farther south, and in New England and New York it is a weed tree which is constantly encroaching on cleared land. These two are the principal food plants and neither is being extensively cut by man. During an outbreak, when the larvae frequently eat all the foliage on the trees over considerable areas, the birches are not killed, even by several attacks in successive years, due to the lateness of the feeding period. The greater amount of feeding occurs during the last of August and September, and at this time of the year the trees have passed through the most active season and are not so severely injured as they would be by a similar attack earlier in the summer. This insect could probably never eliminate its food plant in any given region. It very probably checks the growth of the trees the year after a severe attack, but this check would not be sufficiently great to cause a decrease in the available larval food supply. Another factor that sometimes has some effect on the abundance of a particular insect is the competition for food with other species of insects. The defoliation of the birches in any region early in the summer would very obviously affect the survival of *Bucculatrix*, which feeds late in the season. At present this factor cannot be considered as of much importance. One of the most serious insect enemies of the birch in New England is the saw-fly, *Fenusa pumila* Klug, whose larvae mine the leaves during the entire summer, as there are several generations. Since

this insect confines its work entirely to the new terminal growth, while *Bucculatrix* larvae feed by preference on the older leaves of the tree, the two live together in harmony. There is always the possibility of the last *Bucculatrix* larvae of the brood not having sufficient food, because of the work of the earlier developing part of the brood, and hence being unable to survive. The habit of spending two days in a quiescent state in the molting web increases this danger, for during these two days the foliage on the tree may be entirely consumed. All the observations made in the field indicate, however, that there is no reason to believe that there occurs any decrease in food supply which would have any very important effect in reducing the numbers of this insect even following a year when it was abundant.

No data have been obtained on the effect of climate on the survival of this species. The greatest danger to an insect is during the hibernating period, when severely cold weather sometimes kills off much of the population of certain species. It is a well-known fact, however, that insects which hibernate under the snow are better able to survive extremes of cold than species which hibernate above the snow line. For this reason a very cold winter would not be expected to have a very great effect on the population of the birch *Bucculatrix*. This is an indigenous insect and is inured to the climate of its present geographical range, and the greatest effect of climate on its abundance is probably indirectly through limitations on the distribution of its food plants.

It is not inferred that climatic variations have *no* effect on the population, but rather that climate alone is not responsible for the more or less regular rise and fall in abundance.

The parasites and predaceous enemies of this insect probably account for the increase and decrease in its numbers more than any other one factor. Ten species of Ichneumonoidea and Chalcidoidea have been reared from the larvae and pupae. One of these, *Hemiteles,* is very probably hyper-parasitic, as Viereck (1916) states that all the species of this genus are probably secondary or hyper-parasites. The 10 species with the stage of the host from which they *emerged* are listed below:

	Stage of Host
1. *Bucculatriplex secundus* Viereck Braconidae	pupa
2. *Haltichella xanticles* Walker Chalcididae	pupa
3. *Gelis urbanus* Brues Ichneumonidae	pupa
4. *Cirrospilus ocellatus* Girault Elachertidae	larva (ext. feeding)
5. *Gelis bucculatricis* Ashmead Ichneumonidae	pupa
6. *Mesochorus* sp. Ichneumonidae	pupa
*7. *Pleurotropis bucculatricis* Gahan Entedontidae	pupa
8. *Closterocerus* (*cinctipennis* Ashmead?) Entedontidae	larva (mining)
9. *Derostenus* sp. Entedontidae	larva (mining)
10. *Hemiteles* sp. Ichneumonidae	pupa

* This is a new species the description of which, by Gahan, is published in Psyche, volume 34, June, 1927.

The family names are those used by Viereck (1916). These species are all small and occur singly in the host. The extent to which they parasitize the host varies, of course, from year to year and in different localities.

In the winter and spring of 1924 there were collected 397 cocoons from which there were secured 29 parasites as follows:

Gelis bucculatricis	14	specimens
Bucculatriplex secundus	7	"
Haltichella xanticles	6	"
Hemiteles sp.	2	"

These cocoons were collected from several localities around New Haven, where the host had been abundant in 1923. A large number of pupae died without metamorphosing, and only 152 adult moths were secured from this lot. In 1925 most of the pupae of which records were kept were from larvae reared in the insectary, and the parasitism, therefore, was abnormally low. Nine individuals of *Bucculatriplex secundus* and two of *Haltichella xanticles* were obtained from 352 cocoons. Conditions during the 1925 season of emergence were not normal, as the cocoons had to be kept in the laboratory. The records are not comparable to those obtained a year later. In 1926 there were collected during April and May 209 cocoons in a locality where the larvae had been very abundant the previous season. No collections had been made in this locality during either 1924 or 1925. All these cocoons contained pupae (as later examination showed), and from them were secured 53 parasites and 58 adult moths. The cocoons were kept outdoors in a shaded place until the emergence period was passed, and then those from which no insects had emerged (98 cocoons) were examined. Five contained dead parasites and 93 contained dead pupae. Of the insects which emerged, then, 47.7 per cent were parasites, and of the total number of pupae collected 27.7 per cent were parasitized. The parasites were of the following species:

Bucculatriplex secundus	37	specimens
Pleurotropis bucculatricis	12	"
Haltichella xanticles	1	specimen
Gelis urbanus	2	specimens
Undetermined (escaped)	1	specimen

It is evident that of the insects which emerge from the cocoons the parasites make up a large percentage, and the parasites are better able to survive than the host. Of the 151 non-parasitized pupae, only 58, or 38.4 per cent, produced adults, whereas of the parasitized pupae, 58 in all, 53 or 91.4 per cent produced parasites. The presence of a parasite in a pupa is very easy to determine after three months, as by this time the parasite has consumed most of the host tissue. A parasite could not have been easily over-

looked in the examination of dead pupae. Since these parasites occur singly in the host, the percentages are comparable. There is, of course, the possibility that some of the parasitized pupae died before the parasites had developed far enough to be observed in a dead and desiccated host. The fact that of the 93 dead pupae above mentioned 44 had reached the pre-imaginal stage before dying indicates that this possibility would have no great bearing on the results obtained, for had any parasite been present in any of these, it would have prevented the host from reaching the condition of the pre-imago. It is also true that the parasites are better able to withstand high and low temperatures during the period of emergence than is the host. Three lots of 20 cocoons each were kept at different temperatures, one at 31-33°C., one at room temperature which varied between 18° and 26°, and one at 8-15°. The cocoons were placed in test tubes (50 cc. capacity), 10 in each tube. To serve as a check on the humidity effect, one of the tubes of each lot contained a piece of wet blotting paper which produced a moisture-saturated atmosphere in that tube. The other tube received nothing. All tubes were kept corked except for an interval of about one minute each day when they were opened in the room in order to renew the air supply. The relative humidity of the room averaged 67 per cent, with a variation of 13-14 per cent each side of this for brief intervals of time. The experiment began June 2, 1926. From the cocoons held at room temperature 18 insects were secured, nine from each tube. This represented a normal emergence. Four of these were parasites, all *Bucculatriplex secundus,* and 14 were adult moths. From the cocoons held at 8-15°, two parasites only emerged, one from each tube. One was a specimen of *Bucculatriplex,* and the other was *Mesochorus* sp. From the cocoons held at 31-33° two parasites only emerged, both from the tube containing room air. Both were *Haltichella xanticles.* After being examined July 10th, all the cocoons from which no insects had emerged were removed to the outdoor insectary. Eight adult moths subsequently emerged from the tubes that had been held at 8-15°, four from each tube. After the emergence period was well passed the remaining cocoons were examined. No dead parasites were found, and most of the dead pupae had reached the pre-imaginal stage. Although the number of insects concerned was not large, the parasites were very evidently better able to withstand the extremes of temperature than was the host, for all the parasites emerged under these conditions, but no moths were obtained. The two parasites which came out of the tubes held at 8-15° emerged July 3, and the two from the tubes held at 31-33° emerged June 5 and June 6 respectively. In the case of the latter two, it might be suspected that the difference in development between host and parasites enabled the parasites to complete

the metamorphosis and emerge when the host could not, for they were exposed to the high temperature only three and four days. However, from a third tube set up the same as the others but containing calcium chloride and held likewise at 31-33°, there emerged two parasites only, one specimen of *Bucculatriplex secundus* on June 23, and one specimen of *Pleurotropis bucculatricis* on July 4. The cocoons in this last tube were exposed not only to the high temperature, but also to the desiccating effect of the chloride. No adult moths were secured, and no parasites died before emerging.

In addition to this emergence of parasites from pupae, there is sometimes a considerable parasitism of the mining larvae by *Closterocerus* and *Derostenus*. When these parasites were first discovered, it was thought that they were one and the same species, as they were in the larval stage and resembled each other closely. They are therefore grouped together here. If the mines of the *Bucculatrix* larvae are examined in September, many will be seen to contain the remains of the larva and in addition a very minute parasite larva about .75 mm. in length. September 10 and 14, 1925, there were collected 619 *Bucculatrix* mines in gray birch leaves. Of these, 522 were vacant and showed by the exit hole that the *Bucculatrix* larva had emerged normally. The other 97, or 15.7 per cent, contained each the remains of a *Bucculatrix* larva and one parasite larva belonging to one of the two genera in question. The only exception to this was one mine which contained two parasite larvae. The first of October, 1926, the same locality was visited and 289 mines were collected. The mines this year were much less abundant than in 1925. Of these 289 mines, 100 had been normally vacated by the *Bucculatrix* larvae and 58, or 20.1 per cent, contained parasites. The remainder, 131, contained dead *Bucculatrix* larvae, but the cause of their death could not be determined. It could hardly have been the parasites in question, for the *larvae* of these two species were found in the other mines.

The above figures show that there may be a heavy mortality of the host by the combined attack of the parasites. Of these, *Bucculatriplex secundus* is the most commonly found. Only one locality has been examined for *Derostenus* and *Closterocerus,* and it is not known just how widely spread these two species are. *Pleurotropis bucculatricis, Haltichella xanticles,* and *Gelis bucculatricis* are also rather common. One specimen only has been secured of *Mesochorus* and *Cirrospilus ocellatus.* The former emerged from a cocoon in 1926, and the latter was found in the pupal stage in a molting web of *Bucculatrix. Hemiteles* may be a secondary parasite and hence of no use in checking the reproduction of *Bucculatrix.* There is a possibility that some of the others also are secondary parasites.

The adults of *Derostenus* appear the last of the summer, but the adults of the other parasites appear about the same time that the host adults appear. This indicates that there may be other hosts for some of the parasites. *Bucculatrix secundus* hibernates as a larva in the pupal cuticle of the host. *Derostenus* and *Closterocerus* kill the host larva before it completes the third instar and hibernate as larvae in the mines of the host. The other species hibernate in the pupal cuticle of the host, but the hibernating stage of these was not determined. All the parasites are minute. The Ichneumonoidea adults are about 1.75-2.00 mm. in length, and *Haltichella xanticles* is about the same size. *Pleurotropis bucculatricis* is about 1.5 mm. long, and *Derostenus* and *Closterocerus* are each about .60 mm. in length.

More important as enemies of the *Bucculatrix* larvae than any one of the above species of parasites, and perhaps than all of them combined, are the various species of ants and other predaceous insects which capture the larvae when they descend to the ground to pupate. Ants will not only capture the larvae before the cocoon is well begun, but will also pull a larva out of the cocoon in which it is almost entirely enclosed. In 1925 ants destroyed the entire stock of larvae in the insectary. On one occasion the litter on the ground under a birch which had borne hundreds of larvae was very carefully examined for cocoons after all the larvae had disappeared, and not over 25 entire cocoons were found. A large number of the cocoons were partly completed. This tree had been under observation and no extensive mortality of the larvae on the leaves was noticed. There is no question that most of the larvae reached the ground, and most of these fell prey to their insect enemies before they could pupate. In collecting cocoons in the field in localities where there has been an outbreak of larvae and the trees have been practically defoliated, it is surprising to find relatively few cocoons that are entire and contain pupae.

Although no detailed observations have been made on the activities of birds, Dr. Britton informs me that he has observed certain warblers apparently feeding extensively on the larvae. While there is no question that birds do have some effect on the abundance of these insects, the effect of ants and other predaceous insects seems to be much greater.

The interrelations of host, parasites, and predaceous foes have been very clearly described in the case of the fall webworm by Tothill (1922), whose conclusions are here briefly summarized, and many of the reasons for the occurrence of outbreaks and the following decline in numbers of this insect are applicable to *Bucculatrix canadensisella.* Under normally balanced natural conditions the parasites are most effective and keep the host in an endemic and harmless state for a number of years. The predaceous enemies are also effective, for without their help

the host might increase in spite of the parasites. The combined attack of parasites and predaceous foes reduces the numbers of the host, but at the same time the number of parasites is reduced, for a competitive struggle for food occurs among the species of parasites and among the members of one species. During the last few days of its life in the host, the parasite is much more destructive to the host tissue than at any other time, and although several parasites may start life in one host, which is particularly the case when the host becomes scarce, the only individual that survives is the one which first reaches this rapidly destructive stage, the others perishing from lack of food. The predatory enemies apparently do not discriminate in favor of the parasitized larvae, and this also tends to reduce the number of parasites. Some species of parasites may become locally extinct, and not being strong fliers, do not come in again from the surrounding territory for some years. Any environmental change favorable to the host now gives it an opportunity to increase in the absence of a large part of its enemies, and it soon reaches a stage of great abundance. After a period of years the parasites, which have now found themselves provided with an abundant food supply, increase, and finally, with the aid of the predaceous foes, overcome the host and again reduce its numbers to an endemic state. Over a long period of years the result of these opposing factors is a series of outbreaks following each other at more or less regular intervals. When the host begins to decrease markedly, the parasites also begin to decrease, since they have more difficulty in finding the host, so during the decline of the host population there is not necessarily an increase in the percentage of parasitism. For example, during 1925 the parasitism of *Bucculatrix canadensisella* mining larvae by *Closterocerus* and *Derostenus* was 15.7 per cent, and the following year, in the presence of a very marked reduction in the abundance of mines, the parasitism from these two species was increased only 4.4 per cent.

When the larvae of *Bucculatrix* are abundant there may be expected up to 20 per cent parasitism in the mining instars and an equal percentage of parasitized pupae. To this must be added a heavy mortality due to predaceous enemies. There are also certain undetermined factors, possibly both internal and external, which prevent the development of the insect beyond the pupal stage and cause the mortality of a number of pupae. These last factors are more effective on the host than on the parasite. Aside from the effect of parasites, a considerable number of the mining larvae may sometimes succumb from some cause unknown to the writer. All the factors except parasites maintain a constant attack on the various stages of *Bucculatrix canadensisella,* and when the *Bucculatrix* population begins to decline, the severity of this attack is more keenly felt. A parasite population fluctuates

with a host population and has direct bearing on the periodic abundance of the host but cannot entirely eliminate it, as the parasites decrease when the host decreases.

A species of fungus belonging to the genus *Verticillium* has frequently been found growing on the dead pupae of this insect, and it was thought at first that this might possibly be the cause of these fatalities. Several attempts to inoculate healthy normal pupae with cultures grown on oat agar failed completely. The procedure followed was to make a small opening in the cocoon and expose the pupa within. A drop of water containing a suspension of the spores and mycelium was placed on the pupa, which was then set aside in a petri dish for future observation. Although a number of inoculations were made, in not a single case did an infection of the pupa develop, and it was concluded that the fungus concerned is entirely saprophytic. Several species of *Verticillium* are found on dead insects. I am indebted to Dr. McCormick of the Connecticut Experiment Station for determining this fungus and for carrying out the inoculations.

X. Geographical Distribution

This insect, as far as reports in the literature and information acquired directly from entomologists indicate, is found only in the northern United States and in Canada. Its southern limit is North Carolina, and in Canada it occurs in New Brunswick, Quebec, Ontario, Manitoba, Saskatchewan, Alberta, and British Columbia. Mr. Hutchings, of the Entomological Branch, Ottawa, informs me that it probably occurs up as far as the Yukon. It is recorded as far west as Minnesota in the United States. In Ontario, Quebec, New Brunswick, the New England States, New York, Michigan, Wisconsin, and Minnesota it is very common and sometimes appears in such numbers that the birches are defoliated. On the map (figure 34) is marked with a cross every locality from which I have definite records of the occurrence of the insect.

According to data obtained from Sargent's "Silva of North America" (1896), the four native food plants (the paper, gray, yellow, and black birches) of *Bucculatrix canadensisella* occur over a much wider area than that from which the insect is reported. The region occupied by these birches is shaded on the map. The paper birch (*Betula papyrifera*) is very widespread and is a favorite food plant. It is found almost everywhere within the shaded region on the map, but it is not abundant west of the Rocky Mountains nor south of Minnesota, Wisconsin, Michigan, and New York. The red birch (*Betula nigra*) is not a common tree in northern United States and I have no records of its being attacked by this insect. Its range extends much further south

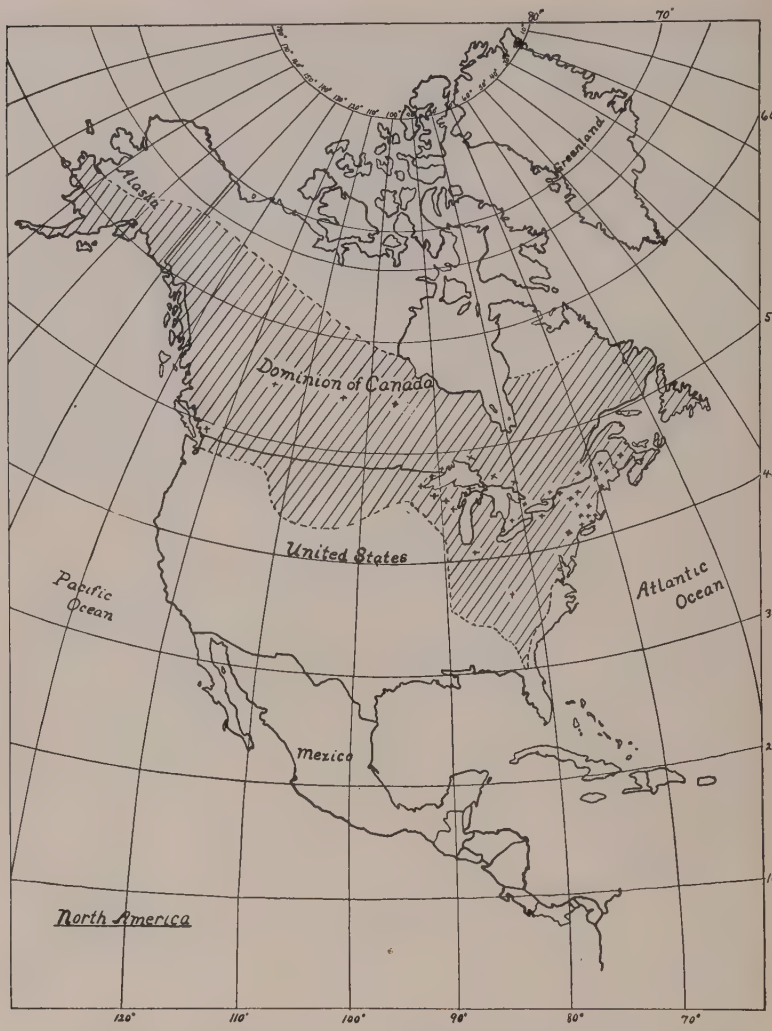

FIG. 34. Distribution of *Bucculatrix canadensisella* Chambers and its food plants. The shaded area shows the distribution of the gray, paper, yellow, and black birches. The crosses indicate localities from which the insect has been recorded.

than that of the other birches. The parts of North America in which these food plants (*B. populifolia, B. papyrifera, B. lutea,* and *B. lenta*) are found corresponds very closely with the boreal and transition zones as outlined by C. Hart Merriam (1898), except for the Rocky Mountain region of the United States. In

Indiana, which is just south of the transition zone, the insects are not plentiful, and in New Jersey they are reported from two counties, Essex and Morris, both in the northern part of the state, and both within the transition zone. In North Carolina adults have been collected in Jackson County. Although the geographical distribution of the birches in the North includes Newfoundland, there are no records of the occurrence of *B. canadensisella* on that island. It is possible that the distribution of this insect coincides with that of the paper, gray, yellow, and black birches, but the map clearly shows that it is most commonly found in the region around the Great Lakes and thence east to the Atlantic Ocean.

That the pupal stage can withstand low temperatures is quite evident, for the region around Port Arthur, Ontario, and the northern shore of Lake Superior very frequently reaches between -20°F. and -30°F. Temperatures would not interfere with the spread of the insect rather far north in western Canada, for the isotherms during the winter run in a curve from Quebec south, and then north through Saskatchewan and Alberta, making the Dakotas, the northern shore of Lake Superior, and the region just north of it much colder than regions directly to the east and west, and this insect is frequently very abundant along the northern shores of Lake Superior. The fact that it hibernates on the ground under leaves and under the winter snow also enables it to endure a very cold climate. It would not be surprising if an examination of white birches during the last of the summer in the northern limits of the range of this tree would reveal the presence of this insect. The southern limit of the insect is also very probably the southern limit of its food plants. This is a very small and inconspicuous moth, and unless it is present in large numbers, it is easily overlooked. As the larval food plants become scattered along the limits of their geographical range, the insect becomes less noticeable. That it has not been reported from more localities is not surprising.

During seasons when it is not very abundant over any great area, the infestations of *B. canadensisella* are often spotted, and a small group of birches may have their leaves completely skeletonized, while one hundred yards away the leaves of others are practically unharmed. This is in all probability due to the fact that the insect flies very little and very rarely goes beyond the shelter of the birch trees.

The insect has probably reached its present geographic range by entirely natural means of spread, for its habits preclude any great distribution by human agencies. It is found on the trees only in the larval state, and then on the leaves only. If birch trees are shipped any distance, transportation always occurs when the tree is dormant and bears no leaves. Birches are cut after the leaves fall, so that there is little probability of cocoons occurring on cut timber. Early records of forest-inhabiting species of

insects are none too common in North America, and *B. canadensisella* was probably very prevalent over the entire area from which it has been reported before Chambers described it in 1875. Even today it attracts no attention except during those periods when it becomes extraordinarily abundant and defoliates the trees.

XI. Effect of Temperature on Development

In view of the fact that temperature seems to be a very important factor in the development of these insects, experiments were carried out to determine the effect of different temperatures on the larva during the period when it was feeding externally on the leaf; that is, during the fourth and fifth instars. The temperatures used ranged from 10° to 35°C., and each temperature was held as nearly constant as possible under the conditions. Those at the lower end of the range, 10°, 11°, 12°, were obtained by using ice-boxes. Incubators were used for 25°, 29°, 34° and 35°, and an incubator was cooled with ice for 14° and 15°; 20°, 21° and 22° were laboratory temperatures. Observations were made at 8.00 A. M., 2.00 P. M., and 10.00 P. M., or as close as possible to these hours, each day, and the temperature and condition of the larvae noted. This gives a possible error of four to five hours in the observations, but in a series of observations this error tends to be compensated. The temperature for any given larval stage is the average of all the readings, and the charted temperatures are those obtained daily by averaging the three temperatures for the day. In all cases a fairly constant daily temperature was held. The temperatures were averaged for each individual larva, and the fluctuations of a degree in either direction made the mean temperatures for different larvae kept in the same location vary slightly. For this reason the groups tabulated under 11° and 12° were both held at the temperature charted on line (c), text figure 35, those tabulated under 14° and 15° were held at the temperature charted on line (d), those under 20°, 21°, and 22° on line (e), and those under 34° and 35° on line (h). In some stages there is a difference of only one degree between groups with very little difference in the duration of the stage at the different temperatures. This is due not only to the fact that one degree would not be expected to show much difference under the conditions, but also to the method of averaging temperature readings. The tabulated temperatures are correct within one-half of one degree; that is, if the temperature for one larva during the fourth instar averaged 20.4°C., that larva was placed in the 20° group. A difference of one-tenth of a degree in the average might throw the larva into a higher group, for if the temperature averaged 20.5°C., the larva was placed in the 21° group.

In view of the fact that the larvae kept in ice-boxes were in darkness, eight larvae were reared in a dark box at the laboratory room temperature, 20°-21°C., as a check on the effect of absence of light. Two of these died in the fifth instar and the other six pupated. The mortality was not exceptional. The mean duration of the fourth instar was 125 ± 5.1 hours, with a standard deviation of 21 hours; the mean duration of the fifth instar was 131 ± 4.3 hours with a standard deviation of 18 hours, and the mean duration of the entire external feeding period was 248 ± 6.8 hours with a standard deviation of 25 hours. These periods are practically the same as the periods of larvae reared at the same temperature in the lighted laboratory, and the absence of light caused no error.

The larvae were reared in individual glass vials as described previously, and the relative humidity was kept constant by wet sand in the vials. The leaves used as food were renewed as often as was necessary for keeping the food material fresh and unwilted. At temperatures of 25° and higher, the leaves were renewed daily; at 14°, 15°, 20°, 21° and 22°, every other day; at all temperatures below 14°, twice a week. The leaves used were all from the lower parts of gray birch trees, that is, the older leaves, and in all but three or four instances were from the same group of trees. Leaves selected were as uniform as possible. All the larvae used in this experiment were obtained from gray birches bordering a field about eight miles north of New Haven. The large number of larvae reared at room temperature (20° and 21°) was due to the fact that a control of each lot of larvae was kept at this temperature.

Four larval periods were considered: (1) the quiescent period in the second molting web; (2) the fourth larval instar, which includes the period in the second molting web; (3) the fifth larval instar up to the spinning of the cocoon; (4) the entire period of life spent outside the mine, which includes the fourth and fifth instars. Although the fifth instar really includes a prepupal period in the cocoon, observations on this period were not possible without disturbing the conditions of the experiment, so this prepupal period was omitted. The actively growing period of the larva is over when feeding ceases, and the omission of the prepupal period does not affect the results. In each case the endpoint is sharply defined.

The chart on page 465, figure 35, gives the temperatures at which the different groups of larvae were kept, and the letter in parentheses at the left of each temperature curve corresponds to the same letter opposite each temperature in tables 4 to 7 and indicates the curve for that temperature group. In the tables are given the number of each larva, the day it began the period

represented by the table, the duration of that period, the mean duration for each temperature group, and the standard deviation for each temperature group (in parentheses after the mean). The temperature at which any larva or group was held, together with the temperature fluctuations during the period, may be ascertained by examining the temperature chart (figure 35). Fluctuations occurred one degree each side of the mean except in a few cases where a brief fluctuation of two degrees is found. The latter cases were so few and the variations in temperature for any one larva were of such brief duration that the results are not affected. Table 8 on page 481 is a condensation of the other tables and gives the data which form the basis for the curves shown in figures 36 to 40. These curves show the relation between temperature and development.

Each figure contains two curves. The curve marked A gives the duration of the period in hours for each temperature within the limits of the curve. The abscissae represent degrees centigrade, and the ordinates, on the left of the figure, hours. The number of degree-hours (developmental units) required for development at any temperature may be calculated from this curve by multiplying time by temperature. If the curve conformed to the formula of a true equilateral hyperbola, the number of degree-hours for each point on the curve would be the same, according to the mathematical definition of the curve, and this constant figure would be the so-called "thermal constant." In no two consecutive temperatures of the experiment were the number of degree-hours equal or approximately equal, and the curves clearly show that no thermal constant exists in the development of the larvae under these experimental conditions. The curve marked B gives the index of development for each degree of temperature. The abscissae are the same as those of the A curve, and the ordinates, on the right of the figure, are the reciprocals of the ordinates of the A curve. Each point on the B curve gives that fraction of the total development which is completed in one hour at that particular temperature. The curve thus gives the rate of development directly and changes in that rate corresponding to changes in temperature. If curve A conformed to the formula of an equilateral hyperbola, the curve B corresponding to it would be a straight line by definition, but A is not an equilateral hyperbola, and B is not a straight line. Where a thermal constant exists, the rate of development curve B is always rectilinear, and if a thermal constant exists for any narrow range of temperatures, within that range the developmental curve is straight. According to the data and the curves, there is no thermal constant over any range of temperatures greater than the error of the experiment. In drawing the curves, the points were plotted for the A curve from the experimental data, and the curve was made to

conform to these points as closely as possible. These points are enclosed in small circles in the figures. The index of development curve B was then drawn to conform to curve A, and the reciprocals of the plotted points in A are enclosed in circles in B. In the absence of a thermal constant, the rate of development of the larvae and the degree-hours required for the completion of any stage must be calculated from the curves directly. No attempt has been made to project the curves beyond the limits of the experimental data.

The lowest constant temperature at which larvae would survive the fourth and fifth instars and pupate was found to be between 10° and 12°C., and the highest temperature was found to be slightly under 34°C. Eight larvae in the first molting web were held at 6°C. [line (a)] 6 days, during which time they did not molt, and four larvae were held at 3°-6°C. [line (a)] 10 days. during which time no molting occurred. The duration of the period in the molting web at 21° is about 40 hours. All twelve of these larvae molted within 29 hours after removal to the laboratory, where the temperature was 20°-21° [line (e)]. Development thereafter was normal. Six larvae in the first molting web were kept at 9°-10°C. [line (b)]. None of these completed development, but three molted within 9 days and lived 50 days. 39 days, and 10 days respectively, after the molt. The other three died in the web without molting. Ten larvae in the second molting web were kept at 9°-10°C. [line (b)], and all molted within 8 days. Four died in 24-45 days, and six pupated in 18-30 days. Controls of all these larvae kept at 20°-21° in the laboratory were normal and had a mortality of zero (25 larvae in all). It is quite apparent that although some development occurs at 9°-10°C., the continuous exposure of larvae in the fourth instar to this temperature is fatal. If the larvae are in the fifth instar, they may or may not complete development, depending on individual hardiness. Ten larvae in the first molting web were kept at 11°-12°C. [line (c)]. All molted within 4 days, 8 completed the fourth instar, and 7 pupated. Ten larvae in the second molt web were held at the same temperature. Eight of these molted within 3 days and pupated. Controls of these two lots were kept at 20°-21° in the laboratory [line (e)], were normal in development, and had a mortality of 2 larvae in 22. At 11°-12°C., development is slow but otherwise is normal. It may possibly be that the fifth-instar larvae are more able to withstand low temperatures than the fourth. Death at 9°-10°C. seemed due to starvation, the cold preventing the larvae from feeding, and even under normal conditions the fifth-instar larvae eat more constantly than do those of the fourth instar. The larvae at this low temperature were always sensitive to touch, and sometimes spun silk threads. It appears as if the first effect of the cold was to stop feeding activity. The

larvae then shrunk in size and finally died from lack of nourishment. The curves (figure 39) of development for the period including the fourth and fifth instars necessarily begin at 12°C., in accordance with the above facts.

At the high temperatures (34°-35°) the effect was somewhat different. The procedure was essentially similar to that described for the low temperatures, and the temperature line (h) in figure 35 depicts the temperature used. This temperature was also fatal to fourth-instar larvae if maintained continuously, but the larvae were able to molt, complete the fourth instar, molt again and begin the fifth instar. None pupated, however. Feeding was very actively carried on until about 12-24 hours before death, and starvation was not a factor to be considered. Fifth-instar larvae molted and pupated without difficulty. The data for the larvae at these temperatures is given in the tables. Since the total feeding period could not be completed at 34°-35°C., the curve of this period stops at 29°C., the highest temperature at which the larvae completed two instars and pupated. At the high temperature, as well as at the low, the fifth-instar larvae seemed better able to endure the adverse conditions and fed more consistently. The lethal high temperature, using the curve as a guide, is in all probability very close to 34°C.

The above data demonstrate that a real threshold of development ("developmental zero") and a real maximum lethal temperature are determinable only when the length of exposure to those temperatures is considered, and that some development may occur at temperatures beyond the lethal high and low (for the entire period) if these exposures are not too prolonged. The difference between that temperature which gives a maximum speed of development and the lethal high is much less than the difference between the same maximum and the lethal low, but development occurs at both extremes. For example, at 9°-10°C., the fourth-instar larvae will ultimately die, but some development will have occurred meanwhile. The same is true of 34°-35° and even higher, for in another experiment an exposure to 37°C. for a few hours caused neither cessation of feeding nor other deleterious effects. In calculating the effect of low temperatures on the life cycle of insects, it has been customary to eliminate all "ineffective temperatures"; that is, all temperatures below a given threshold, this threshold depending, of course, on the insect in question. Theoretically the developmental curve B (figures 36 to 39) should cut the temperature axis at the threshold of development. The corresponding theoretical point on the time-temperature curve A would be at infinity. The curve B, however, shows no marked inclination toward the temperature axis at its lower end, and it would be rash to predict from any data obtained by a time-development study just where it is going to cut that axis. Under natural conditions the temperature fluctuates considerably, and

even though it should rise above or sink below that point at which the larvae could not complete development, some development would probably take place at these extremes of the fluctuations. The fact that the curve A is not a true equilateral hyperbola and the curve B is not a straight line makes it inadvisable to project these curves beyond the experimental data in order to determine theoretical points, and no attempt has been made to determine an absolute threshold of development.

The curves A and B in figures 36 to 39 show clearly the depressing effect of high temperatures on the larvae. According to these figures the maximum rate of development would occur at 30°-31°C., and the experimental data give the maximum rate at 29°C., no experiments being carried out between 29°C. and 34°C. Either side of the 30°-31° point, the rate of development is slower, whether the temperature rises or falls.

The curve B shows the rate at which the speed of development varies and the direction of this variation for each degree of temperature. Beginning at the lowest point in the curve, the rate at which development is speeded up increases with each increase in temperature until at a certain point, the steepest part of the curve, a maximum is reached. At this point fluctuations in temperature have their greatest effect on the development of the larvae. As the temperature increases above this point, the rate at which development is speeded up with the rising temperature decreases until the point of maximum rate of development is reached, 30°-31°C. Any increase in temperature beyond this causes an actual decrease in the rate of development. It is this variation in rate of development which forbids the "summing" temperatures on a "developmental unit" basis.

In all four figures (36 to 39) the curves are sigmoid. In the curves representing the total external feeding period, those temperatures beyond 12°C. in one direction and 29°C. in the other are omitted, due to the non-survival of larvae beyond these points, but the curves take the same general form as the others. The effect of temperature in these experiments was similar for both quiescent and active periods. The shorter periods give the better developmental curves, for as the period lengthens the index of development becomes less numerically, and the curve "flattens," if the system of coördinates remains unchanged. In figure 40 the rates of development as actually obtained have been plotted on logarithmic paper, and the curves give a correct impression of the relative effects of temperature for the different stages. The curves are much more nearly parallel than those plotted on arithmetic paper and show that the effect of temperature on the rate of development of the different stages is somewhat similar.

Due to the small scale on which the curves are drawn, the steepest part of any one of the curves is somewhat difficult to determine by mere examination. However, a calculation of the tangents of

the curves at all temperatures shows that for the period in the second molting web this steepest part lies between 21° and 22°; for the fourth instar, between 23° and 24°; for the fifth instar, between 24° and 25°; and for the total external feeding period, between 23° and 24°. The tangents of these parts of the curves are 1.8855, .6335, .4280, and .2300 respectively. These tangents are calculated for the rate curves as drawn. The lower temperature at which this is found in the period spent in the second molting web may be due to the inactivity of the insect. The temperature relations for the two feeding instars approximate each other fairly well.

A theoretical use of such curves as those marked B in the figures in considering the development of an insect under natural conditions where fluctuating temperatures occur is in the prediction of the time taken to complete a stage of development. In making such calculations, if the mean temperature of a short duration of time, a few hours, for example, be ascertained and the index of development at this temperature be multiplied by the number of hours during which this mean temperature is considered effective, the amount of development completed during this time can be approximately determined. When the sum of these last determinations equals 1, the development is theoretically completed. This is the method proposed by Sanderson (1908) and seems to be more logical than any alternative method. In practice it has been customary to determine the mean temperature for each hour. The method more commonly used in determining amount of development during short intervals of time under conditions of fluctuating temperatures depends on the assumption that the changes in velocity of development vary directly with increases in temperature and that the velocity curve (B in the figures in this paper) is a straight line for a certain temperature range, if not for all temperatures between the "threshold" and the "maximum." With such an assumption there exists a thermal constant within certain temperature limits ("medial" temperature according to Shelford—1926) and the number of developmental units required to complete development is this thermal constant, i. e., the product of time by temperature. If the amount of development completed during a brief interval of time be computed, it will represent a fraction of the thermal constant (*not* of 1), that is, a certain number of developmental units. When the sum of these determinations, the total of developmental units, equals the thermal constant, development is theoretically completed. The fault with this method lies in its uselessness when the velocity curve is not a straight line, and even if part of this curve is assumed to be straight, the method is not good for fluctuations outside of this temperature range. The small scale on which curves are plotted sometimes gives an erroneous impression of rectilinearity.

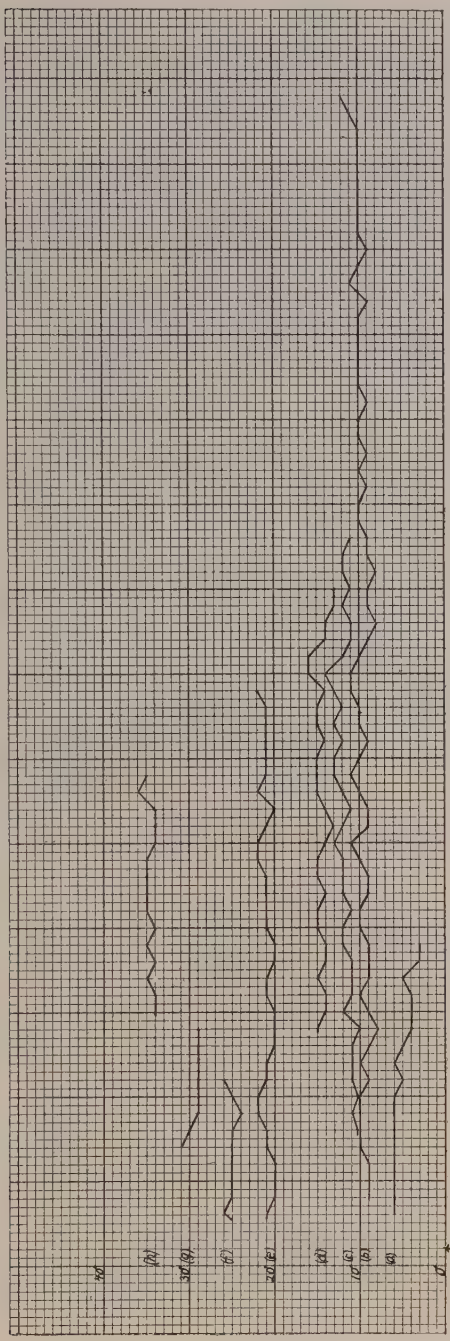

FIG. 35. Chart of temperatures of incubators, etc.

Any method of estimating insect development by averaging widely fluctuating temperatures has its faults, because the development of an insect at a constant temperature is not uniform (Crozier 1926). An approximation is the best that can be attained.

Although it is not the purpose of this paper to enter into a detailed discussion of the effects of temperature on insect life, a comparison of the results here obtained with those of some others is of interest. For further information on the subject the works of Bachmetjew, Sanderson and Peairs, Headlee, Krogh, Glenn, Peirce, Shelford, Payne, and Crozier may be consulted.

Glenn (1922) has attempted to show that in the case of the pupae of the codling moth the rate of development curve becomes a straight line when all the temperatures below a physiological zero (in this case 52°F.) are eliminated from the calculations of the mean temperatures and suitable corrections are made for the high fluctuations above the point of maximal rate of development. The data were obtained under field conditions, and the temperatures are therefore averages of daily fluctuations. However, if the calculations are made of the values the lower points (5.1°, 6.8°, 7.0°, and 8.6° effective temperatures) should have in order to fall into the corrected curve, it is found that in every case the value is lower than the actual data give. The differences are small, but the error probably lies in the fact that some development actually occurred during some of the fluctuations below 52°F., and the elimination of these low temperatures from the calculations caused a slight deviation from the expected results.

Recently Shelford (1926) has used the data of Krogh (1914) on the development of the pupa of *Tenebrio molitor* to demonstrate the fact that within a certain temperature range the rate of development curve may be rectilinear and a thermal constant may be used. In this particular case the range was between 18.5°C. and 28°C. Crozier (1926) has shown that the simple fact that an animal has passed a fraction of its developmental time at a certain temperature does not necessarily mean that that particular fraction of its development has been completed, for the rate of development is not uniform at any one temperature for the entire duration of the period in question, and the curves of development at any two temperatures are not superimposable. Thus, if one-half of the developmental period is passed at a given temperature, more or less than half the development is completed, and when a shift is made to another temperature, there remains more or less than one-half of the development to be completed at the new temperature. Since the curves of development at the two temperatures are not superimposable, the duration of the total period at the two temperatures would depend, among other things, upon which of the two the animals were first exposed to. This fact

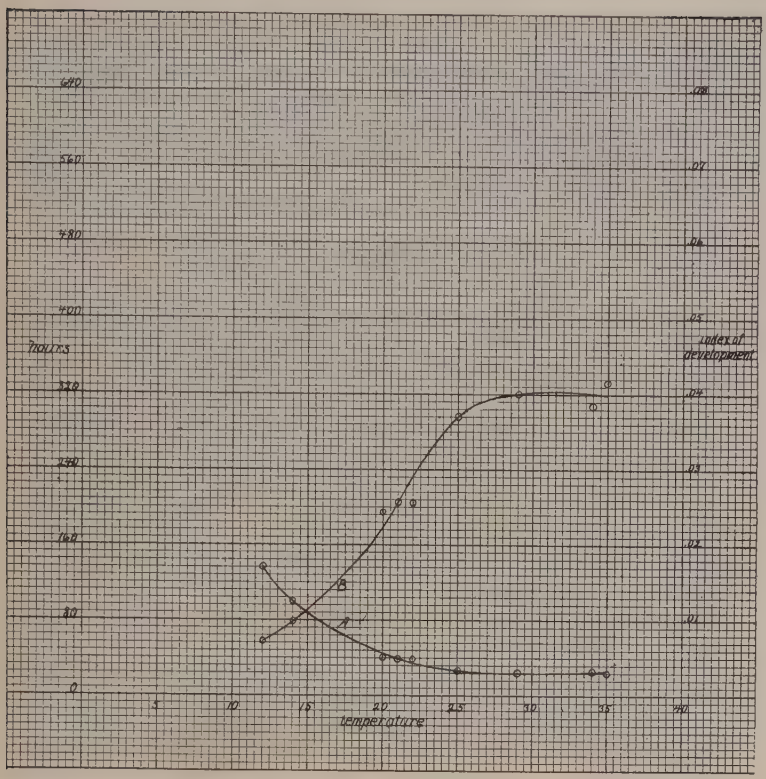

FIG. 36. Effect of temperature on the period in the second molting web. A is the time-temperature curve, and B is the rate of development curve.

alone would throw doubt on the justification of using a thermal constant for any range of temperatures. Moreover, the actual data of Krogh show that the curve is not truly rectilinear even between 18.5° and 28°, but is slightly sigmoid. The calculations were made from Krogh's data, and the last figures are added to show the trend of the curve outside the range in question.

Temperature	Tangent to curve	Angle of curve
18.0 -20.9° C.	.8143	39° 9′
20.9 -23.65	.8291	39 40
23.65-27.25	.8500	40 22
27.25-32.7	.5321	28 1

If the curve were rectilinear between 18.5° and 28°, the second and third tangents at least of those given above should be equal. Moreover, according to Krogh's own statement the Q 10 of the

Van't Hoff formula does not hold for the relation of temperature to development in this particular experiment. Most curves showing this relation have the curvilinear form, and the assumption of rectilinearity for any part of such curves is hardly justifiable.

In attempting to express the relation of temperature to development Crozier and others have brought into use the critical thermal increment of the Arrhenius formula. This formula is as follows:

$$K_2 = K_1 e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

K_1 is the rate of development at the absolute temperature T_1, and K_2 that at T_2; e is the base of the natural system of logarithms, and 2 is the gas constant. μ is the temperature characteristic expressing the critical thermal increment. It has some theoretical significance because it expresses the heat change accompanying the conversion of the participating molecules in the reaction from an "inactive" to an "active" state, and hence corresponds to the sum of the heats of dissociation of the substances taking part in the reaction. The formula gives consistent results for catalytic reactions in pure solutions, and the value of μ is constant over a wide range of temperature.

Blackman (1905) put forward the suggestion that in enzymatic reactions such as characterize biological phenomena, the pace of the entire reaction is governed by that of the slowest reaction of the series composing it, and it is this principle of catenary reactions being controlled, as regards their velocity, by the slowest of the catenary series that Crozier has developed and used in explaining the relations of temperature to growth as well as to other biological processes. Within a certain range of temperatures a certain reaction may be the slowest in the process and hence will govern the speed of the whole, but when the temperature rises above a definite point, another reaction of the chain becomes the slowest and hence the governing one. Within the temperature range governed by one reaction of the series, the temperature characteristic for that reaction is the temperature characteristic for the whole process, and when the second reaction becomes the governing one, a different characteristic, that of this second reaction, governs. In a monomolecular reaction the curve obtained by plotting the logarithm of the rate against the reciprocal of the absolute temperature is rectilinear, as can be readily seen from the formula of Arrhenius. The problem becomes one of getting the value of the critical thermal increment (temperature characteristic) for the process at different temperatures and plotting the above curve. If this is rectilinear, the value of μ is constant, and a temperature constant for the process is obtained.

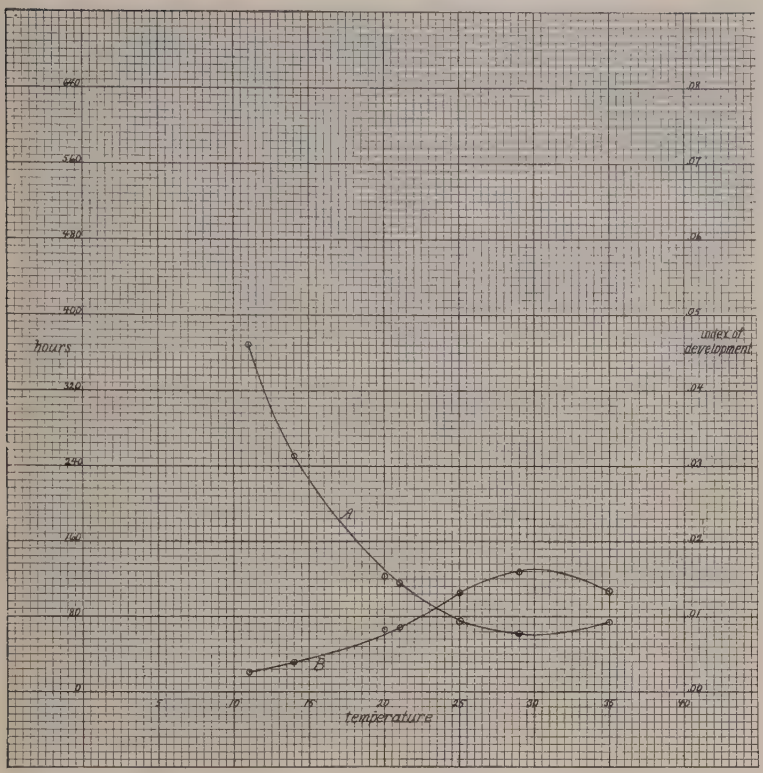

FIG. 37. Effect of temperature on the fourth instar. A is the time-temperature curve, and B is the rate of development curve.

In calculating the value of μ for the development of *Bucculatrix* the period in the second molting web and that of the fifth instar will be considered, as they give two distinct phases in the larval life, and in this particular case contain the data for the most larvae. The values are as follows:

Second Molting Web Temperature	μ	Fifth Instar Temperature	μ
12°-14°	24468	12°-15°	20644
14 -21	23003	15 -21	16637
21 -25	16396	21 -25	14709
25 -29	3431	25 -29	9694
		29 -34	—7793

The values for temperatures above 29° are included simply to indicate the trend of the curve. There is a non-survival of larvae at these high temperatures and the values are of no significance here. A steadily declining value of μ is shown, and if a curve of log rate against reciprocal of temperature were drawn, it would be curvilinear and not straight. The data of Krogh referred to above give the same type of curve, as the following figures, calculated from them, show:

Temperature	μ
13.45°-15.55°	32989
15.55 -17.00	25040
17.00 -18.80	28400
18.80 -20.90	26673
20.90 -23.65	19475
23.65 -27.25	15362
27.25 -32.70	7589
32.70 -32.95	18203

In the case of the last figure in the column the temperature is too close to the preceding to permit any significance to be attached to the value attained. The temperature characteristics, although showing the same tendency of variation, differ in their absolute values from those of *Bucculatrix,* as would be expected. In both cases an increasing temperature gives a decreasing value of μ. The work of Brown (1926) on the development of an instar of various Cladocerans, and that of Bliss (1926) on the prepupal period of Drosophila, however, show a constant value of μ over considerable temperature ranges. Thus Brown finds the following characteristics for Cladocerans:

	Temperature	μ
Simocephalus serrulatus	15° -24.6° C.	16950
	24 -32	4780
Monia macrocarpa	11 -20	28500
	20 -27.6	17210
	27.6-33	7410
Pseudosida bidentata	14 -27.5	19800

Bliss derives the following values for Drosophila:

Temperature	μ
12° -16°	33210
16 -25	16850
25 -30	7100

Crozier (1926) has stated that "there is as yet scarcely sufficient evidence to verify the prediction that the curve relating log velocity of growth to 1/T, when velocity equals reciprocal of

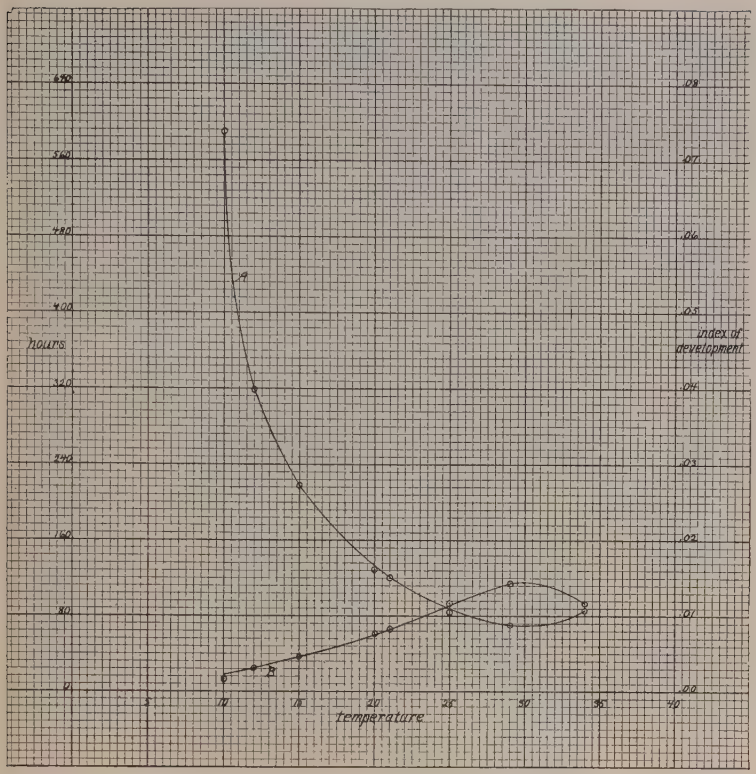

FIG. 38. Effect of temperature on the fifth instar. A is the time-temperature curve, and B is the rate of development curve.

time required to reach a defined stage, should be slightly curvilinear. But there is an indication that growth velocities, where evidenced as constant rates of increase, adhere satisfactorily to the Arrhenius formula; and even when we may quite reasonably expect that an autocatalytic system is involved, the agreement is quite as good as might be desired. The values of the temperature characteristics of growth phenomena are quite varied, yet they cluster quite definitely about the following magnitudes: 7-8,000; 11-12,000; 16-17,000; 20,000; 24,000; 27,000." He brings a considerable mass of evidence to support this view, and for a detailed discussion his works may be consulted. It is simply desired here to compare the values for *Bucculatrix* with those for other animals and to call attention to the fact that different species vary. Heilbrunn (1925) has offered some criticism of the

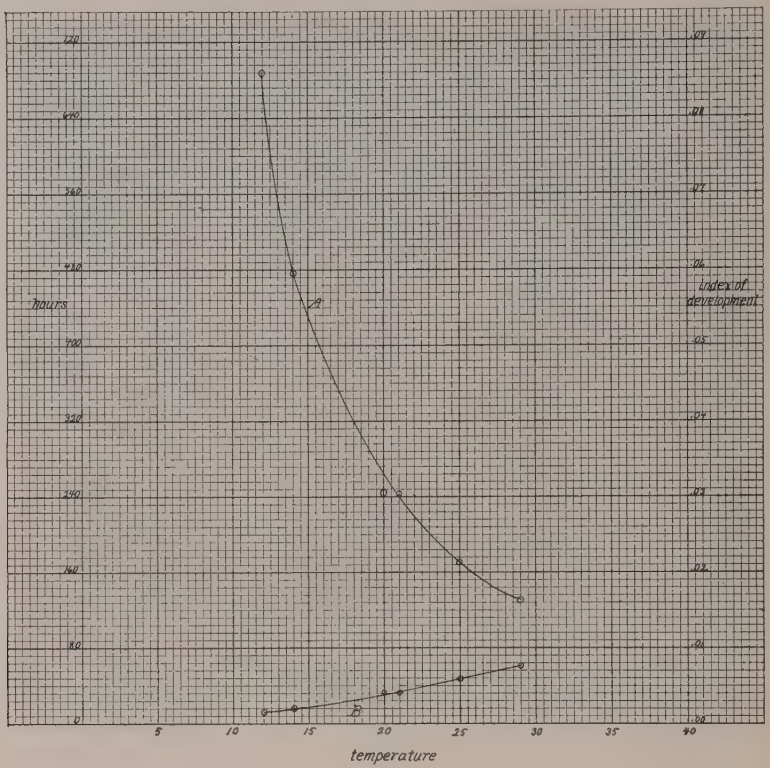

FIG. 39. Effect of temperature on the total external feeding period. A is the time-temperature curve, and B is the rate of development curve.

use of the critical thermal increment of Arrhenius in relation to biological processes of a complex nature and involving several physical as well as chemical reactions, and the identification of basic biological processes by comparing temperature characteristics is open to question.

The effect of temperature on the development of the larvae of *Bucculatrix canadensisella* may be summarized as follows:

The lowest constant temperature at which larvae will complete development is between 10° and 12°C., and the highest temperature is slightly under 34°C.

The curve of the rate of development is sigmoid, and above 30°-31° the temperature has a depressing effect.

In view of the fact that larvae will live for a considerable period of time at 10° and even develop somewhat, although this tempera-

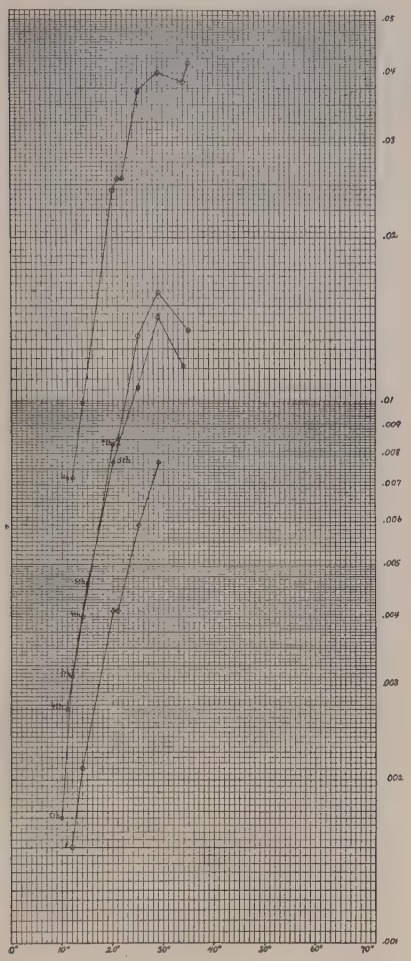

FIG. 40. Effect of temperature on rate of development. W_2 represents the period in the second molting web; 4th, the fourth instar; 5th, the fifth instar; t, the total feeding period. The data are the same as those used in making the B curves in figures 36 to 39 but the plotting paper is arith-logarithmic, and no attempt has been made to smooth the curves. This figure shows the comparative effect of temperature for the different stages.

ture is lethal if continuous, this cannot be considered a physiological zero.

Fluctuations in temperature have their greatest effect on the growth of the larvae when they occur around 21° to 25°.

The temperature characteristic (critical thermal increment) calculated according to the Arrhenius formula is not constant but

steadily declines in value as the temperature increases. This temperature characteristic is not the same for the quiescent premolting period as for the feeding period, and the rate of its change with changing temperature is also different.

TABLE 4. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926

Time in Second Molting Web

Larva No.	Temp. (C.)	Entered web (date)	Hours in web
164	12° (c)	9-21-26	178
165		9-18-26	130
167		9-17-26	136
169		9-24-26	128
170		9-21-26	111
173		9-19-26	138
Mean			137 (20)
251	14° (d)	9-23-26	96
254		9-22-26	112
255		9-23-26	100
256		9-22-26	101
257		9-24-26	95
258		9-22-26	103
259		9-22-26	95
260		9-25-26	104
Mean			101 (5)
122	20° (e)	9-12-26	39
125		9-13-26	54
127		9-12-26	39
128		9-13-26	47
226		9-14-26	41
235		9-12-26	39
131		9- 7-26	40
132		9- 7-26	42
133		9- 6-26	32
134		9- 8-26	39
Mean			41 (5)
123	21° (e)	9-12-26	47
129		9-12-26	33
135		9- 8-26	48
136		9- 7-26	40
137		9- 8-26	48
228		9-11-26	23
229		9-10-26	40
230		9-11-26	23
231		9-11-26	39
232		9-11-26	39
233		9-11-26	39
234		9-13-26	38
281		9-20-26	42
282		9-21-26	40
283		9-21-26	40
285		9-22-26	32
286		9-21-26	40

TABLE 4. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926—*Concluded*

Time in Second Molting Web—*Concluded*

Larva No.	Temp. (C.)	Entered web (date)	Hours in web
287		9-20-26	49
288		9-21-26	40
289		9-20-26	49
Mean			39 (7)
237	22°(e)	9-23-26	34
238		9-23-26	42
239		9-24-26	39
240		9-24-26	40
Mean			39 (3)
110	25°(f)	9- 5-26	31
111		9- 4-26	32
112		9- 5-26	15
113		9- 5-26	28
114		9- 5-26	28
115		9- 6-26	33
116		9- 5-26	30
118		9- 5-26	22
119		9- 4-26	25
120		9- 4-26	25
121		9- 5-26	28
Mean			27 (5)
155	29°(g)	9- 9-26	23
156		9 -9-26	25
158		9- 9-26	25
159		9-10-26	25
160		9- 9-26	23
161		9- 9-26	31
162		9-10-26	24
163		9- 9-26	25
Mean			25 (2)
261	34°(h)	9-17-26	23
262		9-17-26	23
265		9-17-26	33
270		9-17-26	23
Mean			26 (4)
263	35°(h)	9-16-26	24
264		9-17-26	24
266		9-17-26	24
268		9-16-26	24
Mean			24 (0)

TABLE 5. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926

Duration of Fourth Instar

Larva No.	Temp. (C.)	First molt web vacated (date)	Duration of instar (hours)
164	11°(c)	9- 9-26	465
165		9- 7-26	381
166		9- 9-26	343
167		9- 9-26	359

TABLE 5. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926—*Continued*

Duration of Fourth Instar—*Continued*

Larva No.	Temp. (C.)	First molt web vacated (date)	Duration of instar (hours)
168		9- 8-26	319
173		9-11-26	345
Mean			369 (47)
251	14° (d)	9-18-26	231
252		9-15-26	239
253		9-15-26	217
254		9-15-26	281
255		9-17-26	263
256		9-17-26	225
257		9-18-26	240
258		9-15-26	264
259		9-16-26	255
260		9-17-26	281
Mean			250 (21
128	20° (e)	9- 9-26	144
131		9- 4-26	105
132		9- 3-26	143
133		9- 3-26	111
136		9- 4-26	120
281		9-18-26	105
Mean			121 (16)
122	21° (e)	9- 9-26	112
123		9- 9-26	134
124		9- 9-26	119
125		9- 9-26	158
126		9- 9-26	119
127		9- 9-26	112
129		9- 9-26	90
134		9- 4-26	135
135		9- 4-26	144
137		9- 4-26	144
226		9- 9-26	161
228		9- 8-26	81
229		9- 8-26	81
230		9- 8-26	81
231		9- 8-26	97
232		9- 8-26	97
233		9- 8-26	97
234		9- 8-26	144
235		9- 9-26	105
237		9-20-26	106
238		9-21-26	105
239		9-21-26	111
240		9-20-26	145
282		9-18-26	113
283		9-18-26	113
285		9-18-26	130
286		9-18-26	120
287		9-18-26	118
288		9-18-26	113
289		9-17-26	118
Mean			117 (21)

TABLE 5. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926—*Concluded*

Duration of Fourth Instar—*Concluded*

Larva No.	Temp. (C.)	First molt web vacated (date)	Duration of instar (hours)
110	25° (f)	9- 3-26	85
111		9- 2-26	76
112		9- 2-26	69
113		9- 3-26	77
114		9- 3-26	77
115		9- 3-26	94
116		9- 3-26	79
117		9- 3-26	79
118		9- 3-26	71
119		9- 3-26	64
120		9- 3-26	64
121		9- 3-26	75
Mean			76 (8)
154	29° (g)	9- 7-26	71
155		9- 8-26	55
157		9- 8-26	48
158		9- 8-26	55
159		9- 8-26	80
160		9- 8-26	55
161		9- 7-26	71
162		9- 7-26	80
163		9- 8-26	56
Mean			63 (11)
261	35° (h)	9-15-26	77
262		9-15-26	77
263		9-15-26	62
264		9-15-26	63
265		9-15-26	95
266		9-15-26	79
268		9-15-26	62
270		9-15-26	77
Mean			74 (11)

TABLE 6. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926

Duration of Fifth Instar

Larva No.	Temp. (C.)	Second molt web vacated (date)	Duration of instar (hours)
531	10° (b)	9-23-26	567
533		9-24-26	452
534		9-28-26	738
536		9-25-26	587
537		9-24-26	604
Mean			590 (91)
164	12° (c)	9-28-26	249
165		9-23-26	239
167		9-24-26	285
168		9-21-26	388
169		9-30-26	486

TABLE 6. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926—*Continued*

Duration of Fifth Instar—*Continued*

Larva No.	Temp. (C.)	Second molt web vacated (date)	Duration of instar (hours)
170		9-25-26	278
173		9-25-26	284
542		9-25-26	357
543		9-25-26	339
544		9-24-26	259
545		9-24-26	250
546		9-23-26	379
547		9-26-26	403
549		9-23-26	267
550		9-23-26	311
Mean			319 (69)
251	15° (d)	9-27-26	220
254		9-27-26	226
255		9-28-26	211
257		9-28-26	187
258		9-26-26	213
259		9-26-26	229
260		9-29-26	242
Mean			218 (16)
123	20° (e)	9-14-26	120
124		9-14-26	135
126		9-14-26	135
127		9-14-26	152
129		9-13-26	150
230		9-12-26	104
235		9-14-26	107
Mean			129 (18)
122	21° (e)	9-14-26	104
125		9-15-26	120
128		9-15-26	120
131		9- 9-26	115
132		9- 9-26	126
133		9- 7-26	153
134		9- 9-26	93
135		9-10-26	159
136		9- 9-26	135
137		9-10-26	92
226		9-16-26	123
228		9-12-26	135
231		9-12-26	123
232		9-12-26	99
233		9-12-26	99
234		9-14-26	148
237		9-25-26	115
238		9-25-26	170
239		9-25-26	153
240		9-26-26	122
281		9-22-26	129
283		9-23-26	131
285		9-24-26	126
286		9-23-26	94

TABLE 6. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926—*Concluded*

Duration of Fifth Instar—*Concluded*

Larva No.	Temp. (C.)	Second molt web vacated (date)	Duration of instar (hours)
287		9-22-26	115
288		9-23-26	114
291		9-20-26	91
293		9-20-26	120
294		9-20-26	97
295		9-21-26	120
296		9-20-26	97
297		9-20-26	86
298		9-20-26	112
299		9-19-26	104
300		9-20-26	104
551		9-24-26	146
552		9-23-26	99
553		9-23-26	175
554		9-23-26	112
555		9-24-26	116
556		9-23-26	156
557		9-24-26	104
558		9-24-26	178
559		9-23-26	99
Mean			121 (24)
110	25° (f)	9- 7-26	92
111		9- 6-26	97
115		9- 7-26	97
116		9- 6-26	108
117		9- 6-26	80
118		9- 6-26	88
119		9- 5-26	133
120		9- 5-26	75
121		9- 6-26	72
Mean			94 (18)
155	29° (g)	9-10-26	77
156		9-10-26	55
157		9-10-26	80
158		9-10-26	55
159		9-11-26	82
160		9-10-26	81
161		9-10-26	65
163		9-10-26	65
Mean			70 (11)
526	34° (h)	9-22-26	90
527		9-23-26	91
528		9-23-26	96
529		9-24-26	66
530		9-23-26	86
Mean			86 (10)

TABLE 7. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926

Duration of External Feeding Period

Larva No.	Temp. (C.)	First molt web vacated (date)	Duration of period (hours)
164	12° (c)	9- 9-26	714
167		9- 9-26	644
168		9- 8-26	707
169		9- 9-26	803
170		9-11-26	629
173		9-11-26	629
Mean			688 (62)
251	14° (d)	9-18-26	451
252		9-15-26	476
253		9-15-26	456
254		9-15-26	507
257		9-18-26	427
258		9-15-26	477
259		9-16-26	484
260		9-17-26	523
Mean			475 (29)
230	20° (e)	9- 8-26	185
127		9- 9-26	264
128		9- 9-26	264
129		9- 9-26	240
132		9- 3-26	269
Mean			244 (31)
122	21° (e)	9- 9-26	216
123		9- 9-26	254
124		9- 9-26	254
125		9- 9-26	278
126		9- 9-26	254
131		9- 4-26	220
133		9- 3-26	264
134		9- 4-26	228
135		9- 4-26	303
136		9- 4-26	255
137		9- 4-26	236
226		9- 9-26	284
228		9- 8-26	216
231		9- 8-26	220
232		9- 8-26	196
233		9- 8-26	196
234		9- 8-26	292
235		9- 9-26	212
237		9-20-26	221
238		9-21-26	275
239		9-21-26	264
240		9-20-26	267
281		9-18-26	234
283		9-18-26	244
285		9-18-26	256
286		9-18-26	214
287		9-17-26	233
288		9-18-26	227
Mean			243 (28)

TABLE 7. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926—*Concluded*

Duration of External Feeding Period—*Concluded*

Larva No.	Temp. (C.)	First molt web vacated (date)	Duration of period (hours)
110	25° (f)	9- 3-26	177
111		9- 2-26	173
115		9- 3-26	191
116		9- 3-26	187
117		9- 3-26	159
118		9- 3-26	159
119		9- 3-26	197
120		9- 3-26	139
121		9- 3-26	147
Mean			170 (19)
155	29° (g)	9- 8-26	132
156		9- 7-26	118
157		9- 8-26	128
158		9- 8-26	110
159		9- 8-26	162
160		9- 8-26	136
161		9- 7-26	136
163		9- 8-26	121
Mean			130 (14)

TABLE 8. EFFECT OF TEMPERATURE ON DEVELOPMENT, 1926

Duration of Quiescent Period in Second Molting Web

Number of Larvae	Temperature (C.)	Average Time in Web (hours)	Standard Deviation (hours)	Index of Development	Tangent to Curve B
6	12°	137± 5.6	20	.0072	
8	14	101± 1.2	5	.0099	.6770
10	20	41± 1.1	5	.0244	1.3100
20	21	39± 1.0	7	.0256	1.7100
4	22	39± 1.0	3	.0256	1.8855
11	25	27± 1.0	5	.0370	1.2800
8	29	25± 0.5	2	.0400	.8350
4	34	26± 1.4	4	.0385	
4	35	24± 0	0	.0417	.7700

Duration of Fourth Instar

Number of Larvae	Temperature (C.)	Average Duration of Instar (hours)	Standard Deviation (hours)	Index of Development	Tangent to Curve B
6	11°	369±13.2	47	.0027	
10	14	250± 4.4	21	.0040	.2395
6	20	121± 4.5	16	.0083	.4215
30	21	117± 2.6	21	.0085	.4275
12	25	76± 1.5	8	.0132	.6265
9	29	63± 2.5	11	.0159	.1240
8	35	74± 2.6	11	.0135	.3860

Duration of Fifth Instar

Number of Larvae	Temperature (C.)	Average Duration of Instar (hours)	Standard Deviation (hours)	Index of Development	Tangent to Curve B
5	10°	590±27.4	91	.0017	
15	12	319±12.0	69	.0031	.2885
7	15	218± 4.1	16	.0046	.2610
7	20	129± 4.6	18	.0077	.3580
44	21	121± 2.4	24	.0083	.3725
9	25	94± 4.0	18	.0106	.4280
8	29	70± 2.6	11	.0143	.2935
5	34	86± 3.0	10	.0116	.5956

Duration of External Feeding Period

Number of Larvae	Temperature (C.)	Average Duration of Period (hours)	Standard Deviation (hours)	Index of Development	Tangent to Curve B
6	12°	688±17	62	.0015	
8	14	475± 6.9	29	.0021	.1515
5	20	244± 9.3	31	.0041	.1760
28	21	243± 3.6	28	.0041	.1895
9	25	170± 4.3	19	.0059	.2235
8	29	130± 3.3	14	.0077	.1855

XII. Control

The control of these larvae is a very simple matter on ornamental trees. The trees should be sprayed about the middle of August with lead arsenate at the rate of 3 pounds of powder to 100 gallons of water. Add one pound of casein-lime to aid in spreading the poison and making it adhere to the foliage. The larvae feed on the lower side of the leaves, and this side must be covered with the arsenate. Experiments conducted by the writer have shown that if the trees are carefully sprayed there will be practically no feeding by the insects.

XIII. Summary

The history of the genus *Bucculatrix* up to the description of the species *canadensisella* Chambers has been briefly reviewed, and an account has been given of the periodic abundance of this species in North America up to the present. Systematically the genus is usually placed in the family Lyonetiidae.

A brief description is given of the external morphology of the different stages.

There is but one generation a year of *B. canadensisella.* The adults emerge from the cocoons in June and July and oviposit on the leaves of birches. The incubation period of the eggs averages 15 days. The larvae mine in the leaf during the first three instars, the mining period averaging between 24 and 31 days. The last two instars feed externally on the under side of the leaf,

skeletonizing it, and this feeding period averages from 13 to 15 days. The total larval life averages from 38 to 46 days. The cocoon is spun on the under side of debris on the ground, and hibernation occurs in the pupal stage. The last larvae are found in the field the latter part of September. There are five larval instars.

The number of larval instars was determined by applying Dyar's hypothesis to the width of the head capsules.

The principal larval food plants are the gray, paper, yellow, and European white birches. Some feeding on black birch has been observed.

The Hymenopterous parasites, of which ten species have been reared, and the ants and other predaceous foes are the principal factors affecting the abundance of this insect.

The geographical range includes southern Canada and northern United States, the insect being particularly abundant around the Great Lakes and east to the Atlantic Ocean.

Temperature has a marked influence on the development of the larvae. At 10°C and lower, and at 34°C. and higher, they cannot survive. The curve obtained by plotting rate of development (reciprocal of hours taken to complete a given stage) against temperature is sigmoid. Above 30° temperature has a depressing effect. An absolute physiological zero was not obtained because of the ability of the larvae to develop slightly at a low temperature which was fatal if continued a sufficient length of time. There is no thermal constant for any temperature range beyond the experimental error, and the temperature characteristic as computed by the Arrhenius formula steadily decreases in magnitude as the temperature increases.

The use of a lead arsenate spray about the middle of August will protect the trees against injury by the larvae.

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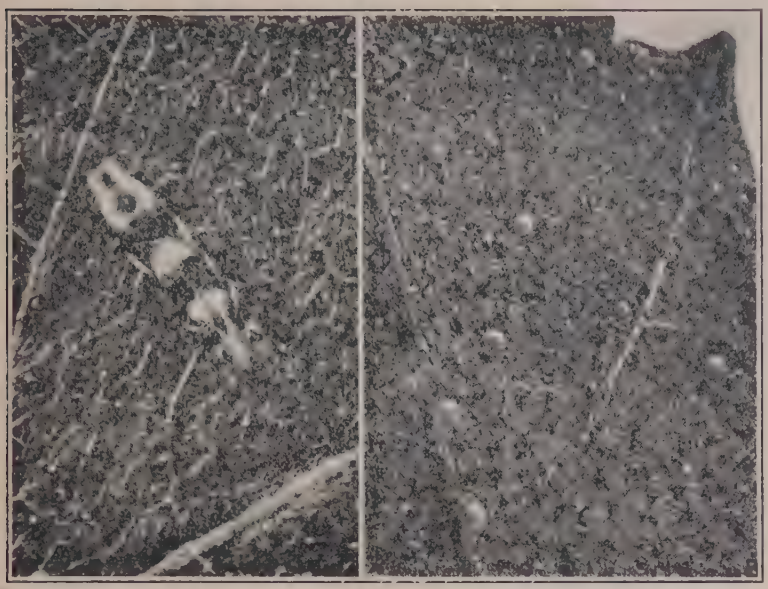
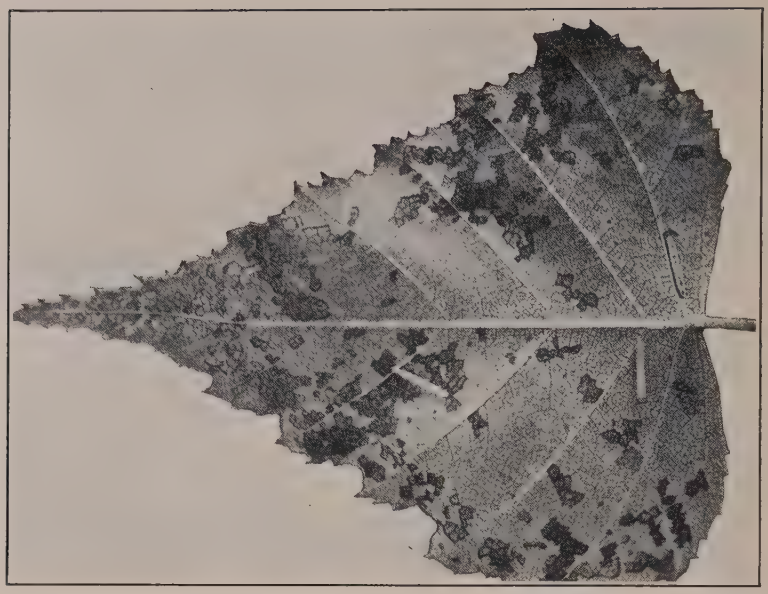


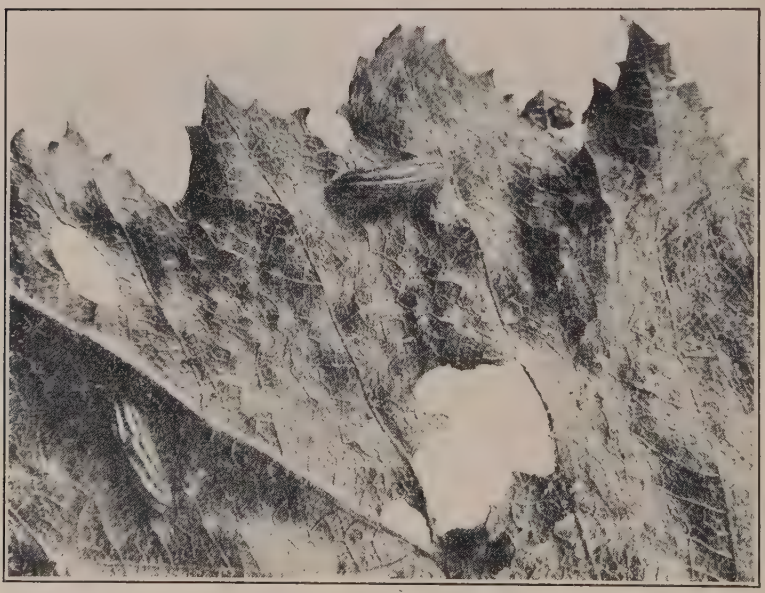
a. Adult (left) and eggs (right) of *Bucculatrix canadensisella* on birch leaves. Adult enlarged eight times, eggs enlarged ten times.

b. Vacant mine of larva in birch leaf (left) and fully grown larva (right) with first (lower) and second (upper) vacant molting webs. Enlarged four times.

BIRCH LEAF SKELETONIZER



a. Larva of *Bucculatrix canadensisella* skeletonizing leaf of gray birch. Slightly enlarged.



b. Cocoons of *Bucculatrix canadensisella* on dead leaf. Enlarged four times.

BIRCH LEAF SKELETONIZER

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ADDITIONAL EVIDENCE AS TO THE INTERCELLULAR FORMATION OF CONNECTIVE TISSUE

By George A. Baitsell

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Read before the Academy April 25, 1927

In a previous communication[1] to the Academy, evidence was presented to show that the connective tissues in the amphibian embryo arise by the direct transformation of an intercellular secretion rather than by a modification of embryonic cytoplasm. Thus, the process is fundamentally intercellular, rather than intracellular. It was also shown in this communication that the intercellular secretion, which constitutes the ground substance of the connective tissues, was of such a nature that, under the influence of various factors—probably both chemical and mechanical in character—it gives rise to the characteristic connective tissue fibers by means of a gradual consolidation and fusion of the minute structural elements of which it is composed. This gradual transformation of the more or less homogeneous, secreted ground substance into a definite fibrous tissue appears, from the morphological standpoint at least, to be identical with that which takes place, under certain conditions, in the transformation of the fibrin of a blood clot into a fibrous tissue, as shown by previous experiments.[2]

Since that time, Harrison,[3] in his investigations on the development of the balancer in Amblystoma, has reached the same conclusion with regard to the formation of connective tissue in this group. He says: "The balancer membrane thus affords a clear proof that connective tissue fibers take origin in an amorphous ground substance, independently of any direct action on the part of the mesenchyme cells." Furthermore, the results from studies[4] on the development of connective tissue in the chick embryo show that essentially the same process obtains there.

For some time investigations have been in progress, under a grant from the National Tuberculosis Association (Medical Research Committee), in an endeavor to determine the origin of the fibrous tissue which, as a rule, forms so abundantly in regions infected with tubercle bacilli, and which undoubtedly plays a very important rôle in the control of the in-

fection and the healing of the affected tissues. Thus it has recently been stated[5] that "The importance of fibrosis in the healing or partial healing of a tubercle cannot be over-estimated. As the connective tissue cells lay down more and more collagen in their peripheries, the intercellular tissue spaces are encroached upon and finally closed, thereby obliterating the only channels of communication between the interior of the lesion and the surrounding tissues. Tubercle bacilli and their soluble products can no longer readily escape from the focus of infection; extension of the infection can no longer take place, and absorption of tuberculin-like bodies can no longer poison the system." It is evident from the above that the question of fibrosis in tuberculosis is a very important one. It is believed that the results herewith presented give clear evidence as to the method of formation of this material in experimentally infected testicular tissues of the guinea pig.

In the present studies[6] on the development of fibrous tissues in tubercular infections, the testes of guinea pigs have been used. The selection of the testis, which has proved to be an almost ideal field for these investigations, was made after an extensive examination of various other organs and tissues. Long[7] had already shown that the testis afforded a very favorable field for his studies on tuberculin. He found that the extent of the degenerative processes, following an injection of tuberculin into the testis, could be used as a direct indicator of the strength of the tuberculin solution. Long also studied the response of the testicular tissues to the living tubercle bacilli and noted that, along with other striking degenerative changes, an abundant formation of fibrous tissue occurred; thus giving evidence that these tissues were adapted for use in the present studies. The exposed position of the testis, making the direct inoculation of any desired region easy; their paired condition, giving a control testis for comparison; and, finally, their unique histological structure, which readily permits following and interpreting the degenerative structural changes that take place, are additional advantages which have been found to be of great importance for these experiments.

Following the general plan used by Long, pairs of male guinea pigs were chosen—the members of each pair being of approximately equal weight. One from each pair, which was termed the reinfected animal, received two inoculations of living tubercle bacilli; the first being a sensitizing inoculation, usually intraperitoneal in which bacilli of low virulence were used; the second, given three weeks later, being a direct inoculation into one testis, of a suspension of highly virulent bacilli. The other animal of each pair, which was termed the control animal, received only the direct testicular inoculation. The response of the testicular tissues in the reinfected animals to the direct inoculation is much more rapid and severe than in the control animals. Later, however, as the infection progresses,

the histological picture in the testes of the two types is apparently identical.

The study of sections shows that the inoculation of living tubercle bacilli into the testis results very shortly in an almost complete degeneration and elimination of the germinal cells from the seminiferous tubules, so that the latter are soon emptied and appear shrunken. Coincident with these changes in the tubules, an abundant exudate appears in the intertubular areas of the testis. In well-preserved material, it is clear that the seminiferous tubules lie, for the most part, in quite close contact so that the intertubular areas are normally quite small, but they soon become greatly enlarged; due, apparently, to the shrinkage of the tubules and the presence of the exudate. Along with the exudate in the intertubular areas varying degrees of cellular infiltration may occur. In many cases, however, large areas of the exudate are practically cell free when first observed.

We may now turn our attention to the origin of the fibrous tissue which, as a rule, is formed so abundantly that within a few weeks after the inoculation, the histological picture of the testis is almost completely changed, the germinal tissues being largely replaced by masses of fibrous tissue. What has been the method of formation? It may be said that the present studies on the infected testicular tissues give clear evidence that the process of fibrosis results from a direct transformation of the exudate present in the intertubular areas. The permanent preparations present a striking and consistent series which is easily interpreted on this basis.

The exudate when first noted appears, for the most part, quite homogeneous or with a fine fibrillation. The later stages show gradual stages in the direct transformation of this material into a typical fibrous tissue, together with the formation of heavy bundles of wavy fibers which stain very beautifully and typically with a Mallory stain. This fibrous tissue encapsulates and infiltrates the tubercles which have been forming in the infected areas, and usually develops in such great abundance throughout the infected areas, as noted above, that the testicular tissues are largely transformed into fibrous tissue.

The transformation of the exudate is not dependent upon direct cellular connections, and is seen to best advantage in intertubular areas in which cells are few in number or entirely lacking. From the morphological standpoint, at least, the process which results in the formation of fibrous tissue from the exudate in the intertubular areas of a testis infected with tubercle bacilli appears to be identical with that which takes place in the development of connective tissue in the embryos of the frog and the chick, and in wound healing in the skin of the adult frog.

Thus it seems safe to conclude from the several lines of evidence now at hand that connective tissue formation is essentially an intercellular

process in which certain cell secretions undergo a direct morphological transformation by a process apparently of the same character as that which takes place in the transformation of a plasma clot.

[1] Baitsell, G. A., *Proc. Nat. Acad. Sci.*, **6**, 1920, p. 77; *Amer. J. Anat.*, **28**, 1921, p. 431.

[2] Baitsell, G. A., *J. Exp. Med.*, **21**, 1915, p. 455; *Ibid.*, **23**, 1916, p. 739; *Amer. J. Physiol.*, **44**, 1917, p. 109.

[3] Harrison, R. G., *J. Exp. Zoöl.*, **41**, 1925, p. 412.

[4] Baitsell, G. A., *Quart. J. Micr. Sci.*, **69**, 1925, p. 571.

[5] Baldwin, Petroff & Gardner, *Tuberculosis, etc.*, Lea & Febiger, Philadelphia; 1927, p. 104.

[6] A preliminary report of these studies was presented at the 22nd annual meeting of the National Tuberculosis Assoc., Washington, D. C., Oct., 1926.

[7] Long, E. R., *Amer. Rev. Tuber.*, **9**, 1924, p. 215; Smith, M. I., *Jour. Med. Res.*, **43**, 1922, p. 45.

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RESULTS OF HETEROPLASTIC TRANSPLANTATIONS OF THE HEART RUDIMENT IN AMBLYSTOMA EMBRYOS

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Communicated May 21, 1927

Adults of the two species of salamanders, *Amblystoma punctatum Linn.* and *Amblystoma tigrinum Green*, differ considerably in size, adult tigrinum salamanders reaching a length of about 25 cm. (in extreme cases 32 cm.) while adult punctatum individuals measure about 16 cm. in length. However, early embryos of the two species are of approximately the same size; at the time of complete yolk resorption the tigrinum embryos may even be smaller than those of punctatum. These two species, therefore, provide suitable material for transplanting an organ rudiment which at the time of operation and during early larval life is of a similar size in the two species but which becomes markedly different in magnitude in later development.

Transplantations of the fore-limb rudiment between the two species have been described by Harrison,[1] who has found that a tigrinum limb transplanted to a punctatum embryo grows much more rapidly than it would have grown if left in place, and that even after ten or twelve weeks, when the tigrinum larva is much larger than the punctatum of corresponding age, the grafted limb is larger than the control limb of either species. On the other hand, the fore limb of punctatum grafted to a tigrinum embryo, although getting an initial start ahead of its normal tigrinum

control, nevertheless ultimately remains very much smaller, and for a long time is smaller than the control limb of the punctatum donor even though the latter is a much smaller animal.

To account for these unexpected results Harrison suggests that in addition to ordinary nutritional factors, two other important factors must be assumed to play a part. First, there exists within the organ itself a factor determining the size to which the organ may ultimately grow; this is greater in A. tigrinum. Second, controlling the rate of growth of the organs of the embryo, there is in the circulating medium a growth regulator; this is apparently greater in A. punctatum. Whatever this regulator of growth rate may be, it seems quite likely that it is in some way associated with energy utilization, of which metabolic rate may serve as an index. This idea has been expressed by Harrison and it is also known from determinations made by Helff[2] that the metabolic rate in punctatum embryos is considerably higher than in embryos of A. tigrinum.

The experiments here reported were done for the purpose of testing these growth relations in an organ particularly vital to the embryo. The experiments also afford an opportunity for studying pulsation rates in hearts exposed to a foreign environment, such as the innervation and blood supply of a different species.

The heart transplantations were done in the usual way at the tail bud stage of development (stage 29),[3] during the period when the heart rudiment consists of lateral folds of mesoderm and a small group of medially placed cells representing the endocardial anlage. This is earlier than the first pulsations and considerably before the time when the nerve supply reaches the heart.

The mortality after such operations is high, but a total of twenty-eight punctatum embryos with hearts grafted from A. tigrinum have been kept alive beyond the stage when the circulation is established and nine have been kept until the beginning of metamorphosis. In all cases acceleration of growth rate began to appear early, a difference in size being evident by the time of complete yolk resorption. The contrast in size gradually became more marked and by sixty days after the operation the grafted heart, compared as to total bulk, was about twice the size of the heart of control punctatum embryos. In general, therefore, the results agree with the results of limb transplants, but by the time of metamorphosis the ratio of size of the transplanted heart to the control punctatum heart was less than at the sixty-day period. Apparently functional regulation had served to bring the organ nearer to the size normal for the host species.

In the reciprocal experiment, involving transplantations of the heart from A. punctatum to A. tigrinum, the transplanted organ grew more slowly than the normal. This result is also similar to the results of limb transplants. In the case of the heart, however, the organ was clearly insufficient

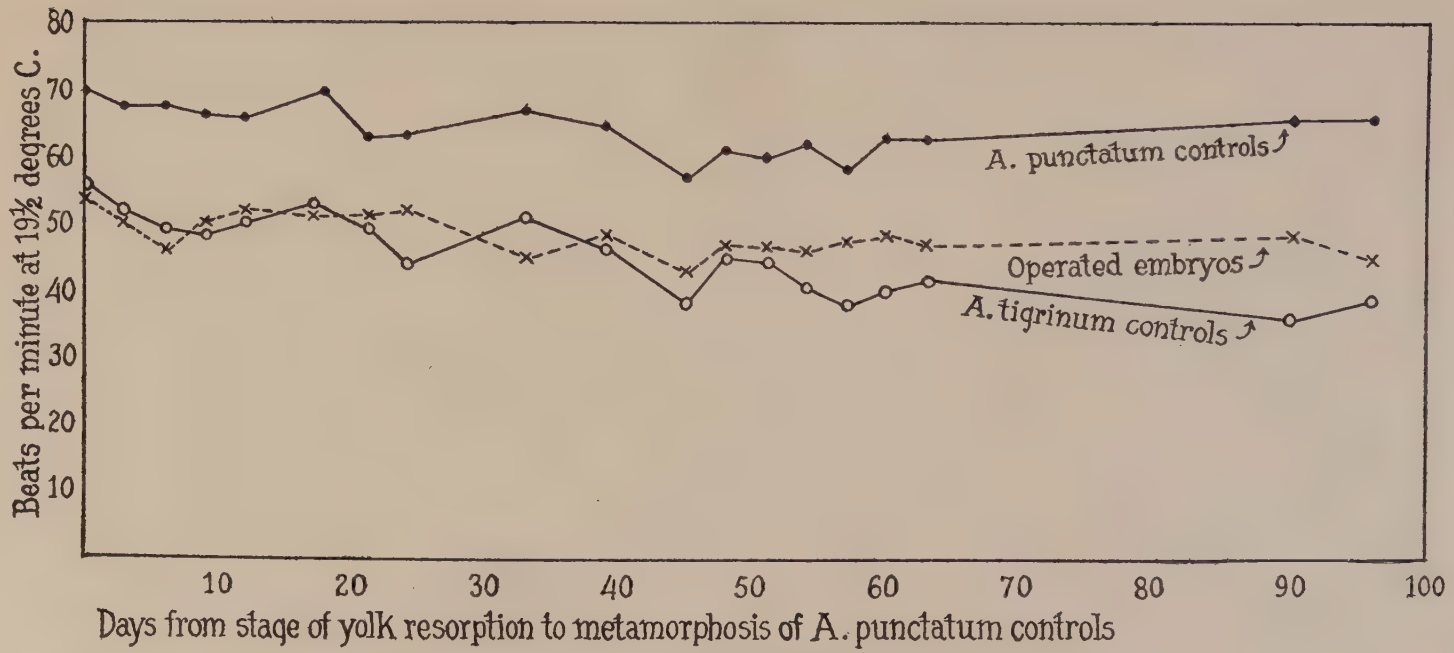
Beats per minute at 19½ degrees C.
80
70
60
50
40
30
20
10
A. punctatum controls
Operated embryos
A. tigrinum controls
10
20
30
40
50
60
70
80
90
100
Days from stage of yolk resorption to metamorphosis of A. punctatum controls

FIGURE 1

for normal function and the embryos became œdematous and lived for only a few weeks.

In addition to transplantations of the organ rudiment as a whole, a few experiments were made in which the posterior half of the heart was removed from a tigrinum embryo and replaced by approximately the posterior half (rudiment of sinus and atrium) from a punctatum embryo. Only four operations of this type were successful, but in these the results were clearly different from the results of transplantation of the organ as a whole from A. punctatum to A. tigrinum. After transplantation of a half, the organ, especially the posterior part, was smaller than normal but was large enough to function during the whole embryonic period, two of the four embryos being kept alive until beginning of metamorphosis.

Pulsation rates have been counted in all heteroplastic graft experiments and these have been compared with pulse rates of animals subjected to control operations (homoplastic grafts) and normal unoperated controls. The normal rate of A. punctatum is considerably higher than that of A. tigrinum, a fact which is interesting in view of the metabolic rate determinations already referred to.

The figure gives a comparison of averages for controls and operated embryos (A. punctatum with heart transplanted from A. tigrinum). The average includes only the records for the nine operated embryos which lived until beginning of metamorphosis. It is evident that the rate for operated embryos is much nearer the rate of the donor than to that of the host species. Apparently the heart of Amblystoma embryos possesses within itself not only the capacity for rhythmical pulsation but also a factor for the rate, and tends to maintain that rate approximately, when placed in a new environment such as the embryo of another species.

Rates in the reciprocal experiments (transplantations of the entire organ from A. punctatum to A. tigrinum) are not given, since the abnormally small heart produced by this cross brought about an œdematous condition of the host. However, a half transplant from A. punctatum to A. tigrinum was sufficient for normal life of the embryo, and the rates in this series were higher than the rates normal for the host species but in the few studied did not approximate the rate of the donor species to as great degree as in experiments of the type illustrated in figure 1. The two series, however, are transplants in the opposite direction and thus far the results do not justify the conclusion that a transplant of the whole of the heart (sinus, atrium, ventricle, and bulbus) approximates the donor rate more nearly than a transplant of posterior half alone, that is, sinus and atrium.

Since the transplantations were made before the time when the nerve supply grows into the heart, the nerves to the transplanted organ must have come from the host system. These nerves are apparently functional in the grafted organ, according to drug experiments made on some of the

embryos. It was found that small quantities of atropine injected through the body wall and over the pericardium had little or no effect on the heart rate. On the other hand, small quantities of pilocarpine definitely slowed the rate and the rate could be brought back approximately to normal by injecting atropine. These results were similar for control and operated embryos. Thus the heart muscle retains approximately its specific rhythm, even though it is under control of the nervous system of a species having a different rhythm.

[1] Harrison, R. G., *Proc. Nat. Acad. Sci.*, 1924, **10**, 69.
[2] Helff, O. M., *Anat. Rec.*, 1926, **34**, 128.
[3] Copenhaver, W. M., *J. Exp. Zoöl.*, **43**, 321.

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THE PREVENTION OF TETANY BY THE ORAL ADMINISTRATION OF AMMONIUM CHLORIDE

W. F. WENNER

From the Osborn Zoölogical Laboratory, Yale University

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Since the pioneer work of MacCallum and Voegtlin (1909), many salts have been used for relief of parathyroid tetany. These investigators, however, were the first to show that the blood of parathyroidectomized dogs contained less calcium than the controls,and that the administration of calcium salts temporarily relieved the tetany produced by parathyroidectomy. Later, Joseph and Meltzer (1910–1911) showed that intravenous injections of sodium chloride was efficacious. Strontium lactate (Swingle and Wenner, 1926)(Dragstedt, 1925) and magnesium lactate (Wenner, 1927), given orally, have been used successfully in the treatment of tetany following the extirpation of the parathyroids in dogs.

The lactate and chloride of calcium were used, but of the two, the chloride appeared to be the more efficacious. Haldane, Hill and Luck (1923) showed that the ingestion of large amounts of calcium chloride causes a change in the reaction of the blood toward the acid side. The calcium passes through the alimentary tract in the form of calcium carbonate and the chlorine, almost completely absorbed, replaces the bicarbonate in the body causing acidosis.

Wilson, Stearns and Thurlow (1915) reported that injection of 0.1 to 0.5 gram of hydrochloric acid brought relief to dogs following the onset of parathyroid tetany. Acid producing salts, such as $CaCl_2$ and NH_4Cl, as shown by György (1922), Gamble and Ross (1923) and Anderson and Graham (1924), are effective agents in the treatment of infantile tetany.

The present work was undertaken with the idea of determining the effect of NH_4Cl on the prevention of experimental tetany and the action of this acid-producing salt on the serum calcium in parathyroidectomized dogs. The series of experiments reported here was completed in the spring of 1926, but publication was delayed a year owing to the fact that this work, together with another paper (Wenner, 1927), constitutes a part of the requirements for the Ph.D. degree, the other requirements for which could not be met until the spring of 1927. During the interim, a paper appeared by Boyd, Austin and Ducey (1926), which dealt with somewhat similar investigations.

Boyd and his associates used seven dogs, three of which were fed on liver and bread, and four on bread and milk. Ammonium chloride in 2 per cent solution was administered by mouth. All three of the dogs on a liver and bread diet died at the end of 26, 20 and 10 days. Three of the four dogs on the bread and milk diet died at the end of 26, 27 and 39 days. One dog survived 197 days. Boyd, Austin and Ducey state that the survival period of dogs receiving ammonium chloride is considerably prolonged but recovery as a rule does not occur.

The writer takes pleasure in acknowledging his indebtedness to Prof. W. W. Swingle for suggesting the problem, and for much helpful advice and criticism.

Six dogs used in this investigation were thyroparathyroidectomized according to the technique described in an earlier paper (Wenner, 1927). The parathyroid glands were identified before and after the thyroids and surrounding fascia were excised. All dogs, with the exception of dog 1, developed violent tetany some time following operation. Great care was taken to see that all of the parathyroid tissue was removed.

Ammonium chloride in five per cent solution was given in 50 to 100 cc. doses twice daily by stomach tube. The solution is retained if given four to five hours before and five to eight hours after feeding.

The data of two of the six experimental dogs are summarized in table 1.

The serum calcium was determined by the Clark and Collip (1925) modification of the Kramer and Tisdall (1921) method. The serum calcium curves of dogs 3 and 5 show the effect of ammonium chloride on the level of the calcium. Dog 2, plate 1, did not develop tetany until after eating a large portion of meat on the thirty-ninth day. Animal 3, figure 1, received no medication until after the first appearance of tetany. Tetany developed two days after operation and following the administration of 5 grams of ammonium chloride, all tetany symptoms disappeared. The next day the serum calcium was found to be 10.6 mgm. or an increase of 3.3 mgm. in 24 hours. Eight days later the dog again developed violent tetany, serum calcium was 6.1 mgm. One and one-half hours after receiving 100 cc. of a 5 per cent solution of NH_4Cl all tetany symptoms disappeared and the calcium rose to 8.5 mgm. per 100 cc. One hundred to 200 cc. of a 5 per cent solution of NH_4Cl given by stomach tube to a dog in violent tetany brought about a recovery within $1\frac{1}{2}$ hours. Figure 5 shows dog 4 in a prostrate condition, all skeletal muscles were twitching, extensor muscles rigid. Figure 6 shows the same animal $1\frac{1}{4}$ hours after receiving 100 cc. of a 5 per cent solution of NH_4Cl.

Animals 1, 2, 5 and 6 recovered permanently after periods of NH_4Cl therapy lasting from two weeks to forty-one days. These dogs were placed on a full meat diet at the end of that time and never again developed tetany. Animal 3, however, was placed on a meat diet, NH_4Cl discon-

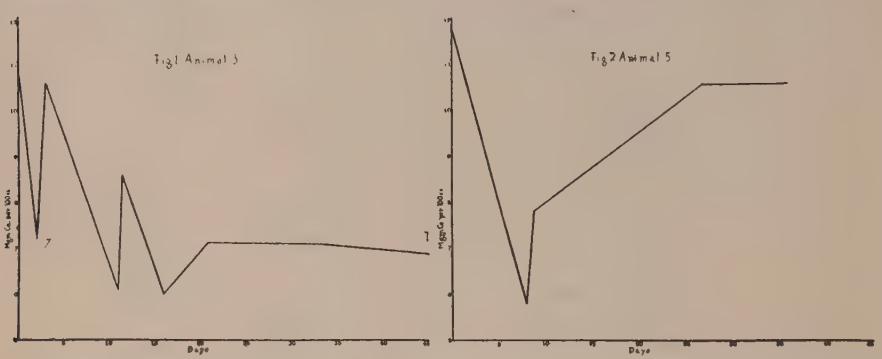
TABLE 1

ANIMAL	DATE	TIME	SERUM CALCIUM	AMMONIUM CHLORIDE GIVEN	REMARKS
	1926		*mgm. per 100 cc.*		
Dog 2					
Adult male	April 10	10:30 a.m.	10.9		Thyroparathyroidectomy
10 kilos		6:00 p.m.		100 cc. 5 per cent solution	
	April 11–14			100 cc. 5 per cent solution	Normal
	April 15		10.9	100 cc. 5 per cent solution	No tetany
	April 16–17			50 cc. 5 per cent solution	Normal
	April 18				No NH_4Cl given. Enema
	April 19			100 cc. 5 per cent solution	Normal
	April 20–23				Good condition. No NH_4Cl
	April 24		9.3	100 cc. 5 per cent solution	Slight twitching of muscles of flank
	April 25–May 17			50 cc. 5 per cent solution	No tetany
	May 18	9:00 p.m.	9.5		Fed large portion of meat
	May 19	2:00 p.m.	6.0	100 cc. 5 per cent solution	Violent tetany, hyperpnea, extensor rigidity
		3:15 p.m.			Recovered. Tetany entirely disappeared.
		6:00 p.m.		100 cc. 5 per cent solution	Normal
	May 20			100 cc. 5 per cent solution	Normal
	May 21				Placed on meat diet. No NH_4Cl given
	May 29		10.3		Normal
	June 12				Normal. Used for other experiments

Dog 6					
Young airedale	May 28		10.2		Thyroparathyroidectomy
9 kilos					
	May 29				No tetany
	May 30				Slight muscle tremors respiration increased
	May 31	5:15 p.m.	6.6		Violent tetany
		5:30 p.m.		100 cc. 5 per cent solution	
		7:00 p.m.		100 cc. 5 per cent solution	All tetany symptoms gone
		9:30 p.m.	10.1		Dog apparently normal
	June 1– 6			200 cc. 5 per cent solution	Normal
	June 7–10			100 cc. 5 per cent solution	Good condition
	June 11				Taken off NH_4Cl therapy. Fed meat
	July 3				Used for other purposes

tinued, June 14—thirty-one days after operation, and fourteen days later died of tetany and pneumonia.

From the results obtained with ammonium chloride it appeared that the anion of strontium and magnesium lactate used in previous experiments, may probably have been equally effective as the cation in preventing tetany. Consequently calcium-free 1 to 5 per cent lactic acid milk was tried. Three parathyroidectomized dogs received daily one quart of lactic acid milk by stomach tube immediately following operation. All three animals died within ten days to two weeks. However, these negative findings do not indicate that lactic acid is entirely ineffective. It will be recalled that Dragstedt and Peacock (1923) placed their experimental animals on the tetany preventing lactose diet four to six days before operation. None of the three dogs treated with lactic acid received any pre-operative treatment.



Figs. 1–2. Serum calcium curves of dogs 3 and 5 showing the effect of ammonium chloride on the level of the calcium.

The use of hydrochloric acid (Wilson, Stearns and Thurlow, 1915) and acid producing substances (Freudenberg and György, 1922; Gamble and Ross, 1923) supported the view that a condition of alkalosis is present during tetany. The recent investigations of Drucker and Faber (1926) on infantile tetany show that infantile tetany is not due to a change in the reaction of the blood in an alkaline direction. Wenner and Muntwyler (1927) have clearly demonstrated that a condition of alkalosis does not exist at any period following parathyroidectomy in dogs. The carbon dioxide content of the plasma, and the hydrogen ion concentration remain within normal range. After recovery from convulsions the pH and alkali reserve of the blood may fall considerably. At this low pH, 7.09, and low CO_2 content, 26.8 volumes per cent, all tetany symptoms disappeared.

In studying the effect of withdrawal of considerable quantities of blood from dogs suffering from tetania parathyropriva, Swingle and Wenner (1926) found that such procedure causes a temporary abatement of all

tetany symptoms coincident with a marked rise in blood calcium within $1\frac{1}{2}$ to 7 hours. Tetany, along with the fall in the calcium, recurs on the

Fig. 3. Animal 2 in violent tetany.

Fig. 4. Dog 2, $1\frac{1}{4}$ hours after receiving 100 cc. of a 5 per cent solution of ammonium chloride.

Fig. 5. Dog 4 in violent tetany.

Fig. 6. Dog 4, $1\frac{1}{4}$ hours later after having received 100 cc. of a 5 per cent solution of ammonium chloride.

following day. Later, Bennett (1926) observed that the removal of one-fourth to one-half of the blood of normal dogs results in a fall of 0.02 to

0.12 pH within four or five hours. On the following day the pH was 0.01 to 0.11 above normal values. It is probable that the rise in calcium produced by bleeding was the direct result of the change in the reaction of the blood toward the acid side.

Binger (1917) was able to show that a reduction in the calcium content of the blood occurs after injections of both acid and alkali phosphates. Tetany accompanied the fall in calcium only when the pH of the phosphate injected was 5.6 or greater. Orthophosphoric acid and acid phosphates produced a decrease in the calcium but tetany did not occur.

The importance of acidity in relation to calcium absorption has been indicated by the interesting work of Steenbock, Hart, Sell and Jones (1923) who found that calcium salts soluble in dilute acids, are absorbed in the intestine because the acid chyme is only gradually neutralized at the pyloric region. A decrease in gastric acidity, affording a poor calcium solution medium and therefore causing a decrease in calcium absorption, has been offered as a suggestion by Babbott, Johnston and Haskins (1923) as a possible explanation for the mechanism in the production of many cases of tetany in infants.

These observations tend to indicate that the administration of NH_4Cl, by increasing HCl metabolism and thereby decreasing the pH of the blood, determines the solubility of the calcium. Greenwald and Gross (1925) owing to their failure to observe an increase in calcium excretion in parathyroidectomized dogs, suggested that calcium is precipitated in the tissues as calcium phosphate, which in the absence of the parathyroid hormone becomes less soluble. With the increase in the acid radicle it is possible that this salt is brought into solution.

The permanent recovery after thirty to forty days' treatment with ammonium chloride was certainly not the result of any failure to remove all parathyroids at the time of operation. Out of nearly two hundred dogs operated on during the past few years only three have failed to develop tetany following complete removal of the parathyroid glands. It is true that accessory parathyroids occur in a large percentage of cats; Nicholas and Swingle (1924–25) found accessory parathyroids in 35 per cent of these animals, but in dogs, however, accessory glands occur less frequently. Marine (1914) states that only 5 to 6 per cent of dogs were found to possess them. Of the forty dogs used as controls, and for experiments on potassium, sodium and cadmium lactate, lactic acid and lactic acid milk, all died of tetany within two weeks. The same careful technique was used for all operations.

The mechanism of the readjustment that takes place within the body after forty days' treatment with strontium lactate (Swingle and Wenner, 1926), magnesium lactate (Wenner, 1927) and ammonium chloride has been discussed in an earlier paper (Wenner, 1927) where it was suggested

that the mechanism of adjustment may involve 1, the hypertrophy of accessory parathyroid tissue, or 2, the taking over of the function of the parathyroid glands by other organs. However, it should be stated that no evidence exists at present in favor of either suggestion in so far as dogs are concerned.

SUMMARY

1. Ammonium chloride administered orally is efficacious in the prevention of tetany in parathyroidectomized dogs.

2. Dogs in violent tetany recover within one and one-half hours after receiving 100 to 200 cc. of a 5 per cent solution of ammonium chloride.

3. Thyroparathyroidectomized dogs receiving daily administrations of ammonium chloride for thirty to forty days become readjusted to the loss of the parathyroids and may be placed on a meat diet without ill effects.

4. Ammonium chloride probably exerts its action by rendering the blood more acid thereby producing a rise in the serum calcium and a disappearance of the symptoms of tetany.

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ADAPTATIONS OF THE BATHYPELAGIC NEMERTEANS

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Of all the groups of organisms which recent investigations have discovered in the deep oceans, probably none have become more widely divergent from their littoral relatives and more particularly adapted for a free-swimming life far beneath the surface of the water than have the nemerteans.

The object of this paper is to call attention to some of these special adaptations, but with particular reference to the material collected by the U. S. F. C. S. *Albatross* in various parts of the Pacific Ocean. The results of a detailed study of this material have been published in monograph form during the past year (Coe, '26).

Although the total number of described species of nemerteans is less than six hundred, they are very widely distributed, being found on the bottoms and along the shores of all the oceans, less commonly in fresh water, and occasionally in moist earth and even in moist cavities on trees. They are most numerous along the sea-coasts of Europe and North America, but relatively few species extend along the bottom into the deep oceans which lie beyond the continental shelves. In the open oceans they are represented by a highly specialized group of the Hoplonemertea, constituting the tribe Pelagica, with peculiar adaptations for slowly swimming or floating idly in particular water layers which lie at considerable depths. It is with this bathypelagic group that this paper deals.

Forty-seven species of these highly modified forms have been described. These have been grouped in nine families and twenty-five genera. The members of each

family naturally have characteristics peculiar to themselves, but many of them have a great amount of gelatinous tissue, or parenchyma, separating the internal organs and giving the worms a very low specific gravity and in many cases a gelatinous consistency and translucency so commonly found in pelagic organisms (Fig. 3). Ocelli, cerebral sense organs and the other special sense organs of their littoral relatives are rudimentary or entirely wanting and excretory organs have not been discovered in any species.

In external appearance the bathypelagic species show great diversity in size and shape as well as in their degree of translucency and in their coloration. In some of the species the sexually mature worms do not exceed 6 to 10 mm in length; most of the other species range from 20 to 40 mm long, while the largest known specimen of *Dinonemertes investigatoris* was 203 mm long, 56 mm wide and 15 mm thick. The bulk of an individual of the largest form is thus some ten thousand times as great as that of the smallest, comparable perhaps to the relative sizes of the ox and the mouse.

The shape of the body is generally quite different from that of most of the littoral species, the latter being as a rule very slender and often ribbon-like and adapted for burrowing in mud or sand or for creeping along submerged objects. A few of the littoral forms, however, are relatively short, broad and flattened and adapted for creeping beneath stones and in narrow crevices. The bathypelagic forms are nearly all broad and flat (Figs. 1, 3, 4) and adapted for maintaining themselves suspended horizontally at great depths with a minimum of muscular effort.

Correlated with the flattening of the body are frequently lateral outgrowths of the body walls in the form of very thin lateral margins, often forming lateral keels or rudimentary horizontal fins. In many forms, also, the posterior extremity is broadened and extremely flattened to form a well-developed caudal fin, giving the worm a

remarkably fish-like appearance (Fig. 4). In only a few species is the body relatively slender and narrowed at both ends, as is so generally the case in littoral nemerteans.

A few of the more characteristic forms of the body are shown in Fig. 1. It will be observed how well they are

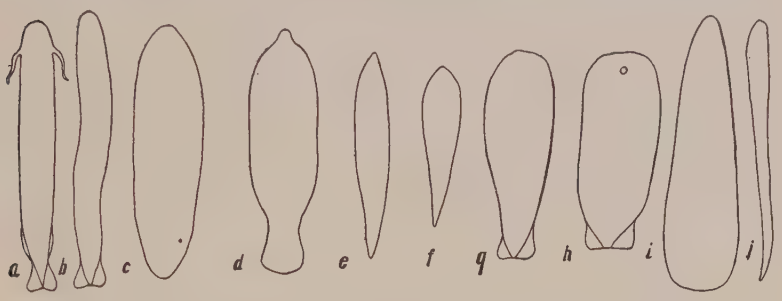


FIG. 1. Outlines of the bodies of several species of bathypelagic nemerteans, showing their adaptive configuration for swimming or floating. *a, Nectonemertes mirabilis* Verrill, male; *b, N. pelagica* Cravens and Heath, female; *c, Planktonemertes agassizii* Woodworth; *d, Neuronemertes aurantiaca* Coe; *e, Protopelagonemertes hubrechti* Brinkmann; *f, Cuneonemertes gracilis* Coe; *g, Pelagonemertes brinkmanni* Coe; *h, Proarmaueria pellucida* Coe; *i, j,* dorsal and lateral views of *Dinonemertes investigatoris* Laidlaw.

adapted for holding themselves in a horizontal position by undulatory movements of the posterior end of the body.

Since the maintenance of their floating position requires more or less continuous undulatory movements of the body it is not surprising to find the musculature highly specialized for this purpose. Instead of having the thick cylinders of circular and longitudinal muscles characteristic of the littoral species, the circular musculature is reduced to a very thin layer of fibers, while the longitudinal musculature is developed into two great horizontal bands, one of which lies on the dorsal side of the body and the other on the ventral side, comparable to the pair of vertical bands which furnish the principal motive power of the fish. The alternate rhythmical contraction of dorsal and ventral muscular bands enables the worm to swim with undulatory movements, the caudal

fin, when present, being the effective organ of propulsion. The degree to which this caudal fin is developed in various species is shown in Figs. 2, 3, 4.

In those species in which the body reaches its extreme degree of thinness there is a great increase in the muscles which pass directly from the dorsal to the ventral side of the body between the intestinal diverticula. The contraction of these muscles brings the dorsal and ventral surfaces of the body closer together and thus makes the worm still more ribbon-like by decreasing its thickness and increasing its length. This dorsoventral musculature reaches its highest degree of development in the posterior part of the body, sometimes producing the extremely thin lateral margins or horizontal fins to which reference has been made (Figs. 2, 4).

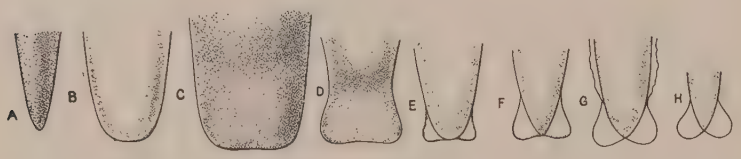


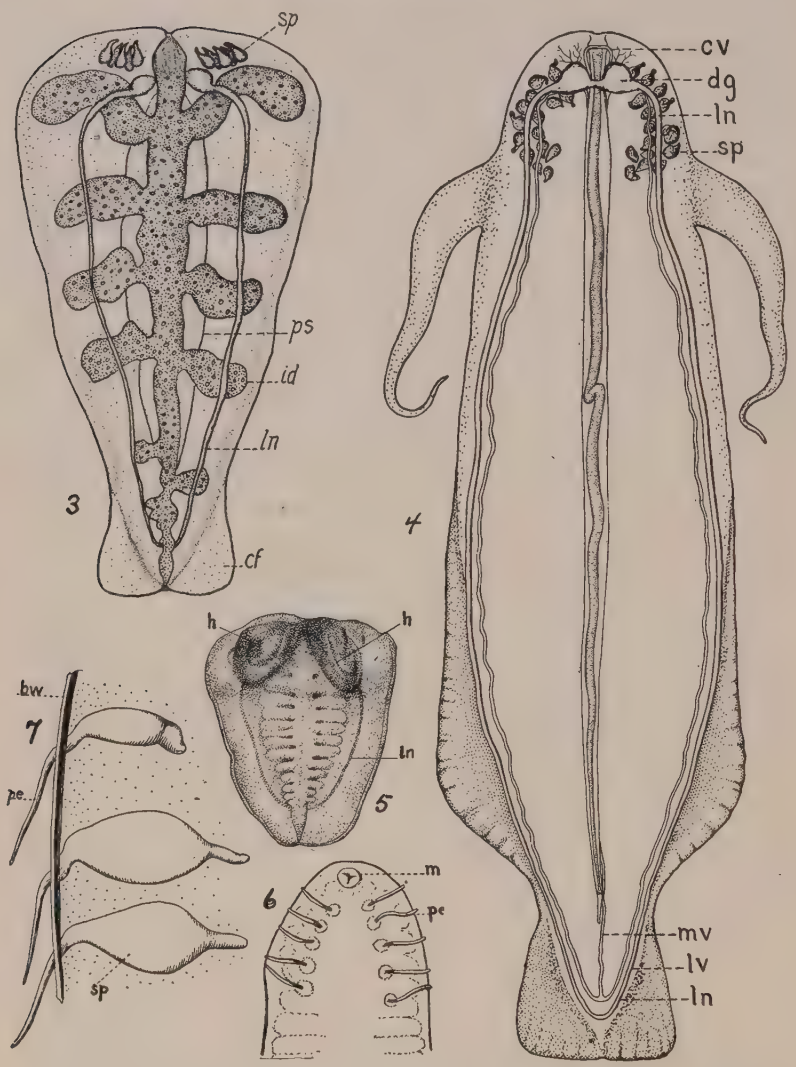
FIG. 2. Diagrams of posterior extremities of the bodies in eight genera of pelagic nemerteans, showing the extent of development of the caudal fin. *A, Cuneonemertes gracilis* Coe, with slender, subcylindrical posterior end; *B, Planktonemertes agassizii* Woodworth, with flattened, rounded extremity; *C, Dinonemertes investigatoris* Laidlaw, with extremely flat, truncate end; *D, Neuronemertes aurantiaca* Coe, with flat, truncate extremity, but without distinct caudal fin; *E, Proarmaueria pellucida* Coe, with slightly developed caudal fin; *F, Pelagonemertes brinkmanni* Coe, with fairly well differentiated caudal fin; *G, Nectonemertes mirabilis* Verrill, with highly developed caudal fin and pair of lateral fins; *H, Balaenanemertes* sp., with extreme development of caudal fin.

A few forms are provided with appendages in the form of a pair of tentacles near the anterior end of the body (Figs. 1, 4). These tentacles, which are lateral outgrowths of the body walls, occur only in the males of *Nectonemertes* but are found in both sexes of *Balaenanemertes*. In the former genus they reach their full development only with the sexual maturity of the male and are therefore to be looked upon not only as aiding somewhat in the balancing and floating of the body, but

more particularly as appendages for grasping and holding the female until insemination has taken place. For these worms form a sparse population in the depths of the open oceans and any device for holding the sexes in contact, when they chance to meet, until after the eggs have been fertilized may well be looked upon as an important adaptation. In *Plotonemertes* a similar result seems to be accomplished by means of a pair of adhesive organs on the ventral side of the body (Fig. 5). Other curious adaptations in this direction are the situation of the spermaries in or near the head or even in front of the brain in some species (Figs. 3, 4, 6) and specialized musculatures for the forcible discharge of the spermatozoa. In *Phallonemertes* the spermaries are not only provided with a spiral musculature, but the sperm ducts project beyond the body as slender muscular tubes (Figs. 6, 7) which conceivably function as copulatory organs.

In addition to the insurance of fertilization, provision is made for the production of a small number of embryos, each with maximum nutrition. This is accomplished through the absorption of the greater number of the primitive ova to form relatively few of larger size. Not only is the number of ovaries greatly reduced, but only a single one of the numerous primitive ova in each ovary reaches maturity. In one form, *Proarmaueria pellucida,* hermaphroditic gonads occur in addition to ovaries, a condition which would seem to offer certain advantages in a very sparse population.

Many of the species are more or less translucent and some are largely transparent, with only the digestive system and the gonads more opaque and pigmented (Fig. 3). The pigments are of two classes, many species bearing numerous brightly colored oil globules in the walls of the digestive system, while others have in addition a more diffused pigmentation of the whole body. The colors are frequently very bright, shades of yellow, orange, red and scarlet predominating. It must be remembered, however, that since none of the sun's light



FIGS. 3–7. Adaptations of several pelagic species. Fig. 3. *Pelagonemertes brinkmanni* Coe, indicating the gelatinous consistency of the body, with the deeply colored digestive system showing through the translucent tissues. The spermaries (*sp*) are grouped in two small clusters in front of the brain. Fig. 4. *Nectonemertes mirabilis* Verrill; adult male with fully developed tentacles and cephalic spermaries (*sp*); note fish-like configuration of body, with horizontal and caudal fins. The slender proboscis is shown in the proboscis sheath. Fig. 5. Posterior portion of body of male *Plotonemertes adhaerens* Brinkmann, with pair of glandular organs (*h*), conceivably used in adhering to the female until insemination is completed. Fig. 6. Outline of anterior end of body of male *Phallonemertes murrayi*

rays of these colors reach the depths at which many of the species live the coloration which appears when the animals are brought to the surface must not be assumed to indicate a color adaptation.

In the earlier collections of bathypelagic organisms, which were taken in a dredge or in a net hauled vertically from the bottom, it was impossible to determine the depth from which any given specimen was obtained. By the more recent methods, however, where several nets are placed at different points on a single wire and drawn horizontally at various depths simultaneously the habitat of any species can be determined with considerable accuracy. By such means the approximate bathymetrical range of several species has already been determined. *Nectonemertes mirabilis,* for example, was found by the *Michael Sars* expeditions at numerous stations in the North Atlantic from off the continental shelf near Newfoundland to the similar shelf off the coast of Ireland. Brinkmann ('17) has plotted the distribution of these specimens and finds that the species is more or less closely confined to a water layer having a salinity of somewhat less than 35 per cent. and a temperature of about 4° C. Since this layer is closer to the surface in the western Atlantic than it is at points nearer the coasts of Europe it is to be expected that the depth at which the species is most frequently found will be correspondingly greater towards the east. The data indicate that such is the case, the depth of greatest frequency being about 1.333 meters in the western Atlantic.

Most of the *Albatross* specimens were taken in nets drawn at a depth of about 600 meters beneath the surface in regions where the distance from the surface to the ocean's floor was 2,000 meters or more. We have, unfor-

Brinkmann, showing the five pairs of external sperm ducts (*pe*) projecting from the ventral surface. Fig. 7. Diagram of three spermaries of same species, showing relation of sperm ducts (penes, *pe*) to body wall (*bw*). Other lettering: *cf,* caudal fin; *cv,* cephalic vessels; *dg,* dorsal ganglion; *id,* intestinal diverticulum; *ln,* lateral nerve; *lv,* lateral vessel; *m,* mouth; *mv,* median dorsal vessel; *ps,* proboscis sheath. (Figs. 5, 6, 7 after Brinkmann, '17.)

tunately, no proof as to bathymetrical range of any of these species, and it is quite possible that other species or more numerous specimens of the same species might have been secured if the nets had been lowered to a depth of 1,000 or 1,500 meters.

The absence of appropriate water layers in seas and bays along the continental shelves explains the absence of bathypelagic nemerteans in those regions, while the continuation of these layers beneath the equator allows their free dispersal between the northern and southern hemispheres as well as through all southern oceans. Thus one species, *Dinonemertes investigatoris,* has been found in both the North Atlantic and Indian oceans, *Nectonemertes minima* in the Atlantic from 57° N. to 35° S., *N. pelagica* in the Pacific from 52° N. to 12° S., while *Pelagonemertes rollestoni* extends from the equator in the Atlantic, through the deep water off the western coast of Africa and thence to 4° N. in the Indian ocean and to 50° S. at the region where the Indian and Pacific oceans meet. And it is not improbable that the limits of even this great known range will be extended by future explorations. For up to the present only a minute fraction of the waters of the globe have been explored with apparatus suitable for the collection of bathypelagic organisms.

The bathypelagic nemerteans, therefore, are truly mesoplanktonic organisms, swimming slowly to and fro or floating idly far beneath the surface of the open oceans. For such a life they are admirably adapted, for they are composed so largely of gelatinous tissue that their specific gravity is very low and the configuration of their bodies is such that their position in the depths can be maintained with a minimum expenditure of energy.

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Studies on some Amoebae from the termite Mirotermes, with notes on some other Protozoa from the Termitidae.

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With Plates 22–6.

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I. Introduction.

During a visit to Barro Colorado Island in the Canal Zone in the summer of 1925, observations in many termites of the family Termitidae, most members of which according to previous examinations contain no Protozoa, resulted in the discovery of small flagellates and amoebae in several species. The most numerous and striking amoebae occurred in Mirotermes hispaniolae Banks and Mirotermes panamaensis Snyder, termites which have wood-boring and wood-feeding habits similar to those of the lower families.[1] Previously Cleveland (1923) had found Protozoa in alcoholic specimens of these two species of termites which had been collected from the same locality and preserved in the collections of the U.S. National Museum, but since the Protozoa were in poor condition he was unable to classify them. However, it is probable that they were the same as the amoebae described below.

Of these amoebae three species occurred in the intestine of every individual from one colony of Mirotermes hispaniolae which was examined, and two in the same way in four colonies of Mirotermes panamaensis. One in each termite is large in size, the others smaller, and they differ sufficiently in the two hosts to be readily distinguished, at least when stained—much more readily than their hosts. Indeed, they may serve as a means of separating the species of insects, which morphologically resemble each other closely.

Minute amoebae in such small numbers that it was necessary to search for them carefully were found in Amitermes beaumonti Banks, Amitermes medius Banks, Subulitermes kirbyi Snyder, and Cornitermes acignathus Silvestri. The other species of Termitidae examined in the Canal Zone in which no amoebae were discovered were Cylindrotermes nordenskiöldi Holmgren, Armi-

[1] Dr. Snyder is the authority for this statement. The colonies of Mirotermes panamaensis which provided material for this work lived in firm stumps and logs; that of Mirotermes hispaniolae in a living tree.

termes chagresi Snyder, Nasutitermes cornigera Motschulsky, Nasutitermes pilifrons Holmgren, Nasutitermes ephratae Holmgren, Obtusitermes panamae Snyder, a species of Microcerotermes, Subulitermes zeteki Snyder, Orthognathotermes wheeleri Snyder, and a species of Anoplotermes. In the last two of these, and in all those, including Mirotermes, in which amoebae occurred, minute flagellates were present in small numbers.

Several observers have examined termites of the family Termitidae for Protozoa with negative results, but I am aware of only one other positive record. From the examination of alcoholic material Cleveland (1923) recorded Protozoa in a species of Nasutitermes from the Belgian Congo, as well as in these two species of Mirotermes.

Observations of living material were made in the field by removing the intestinal contents and mixing them on a slide with a drop of diluted Locke's solution. Besides this study, a quantity of smears on both cover-glasses and slides was prepared, fixed in Schaudinn's, Bouin's, or Flemming's fluids, and preserved in 85 per cent. alcohol. This material was stained after my return in Heidenhain's or Delafield's haematoxylin with or without acid fuchsin, eosin, or Biebrich's scarlet counterstain; in alum carmine; or in methyl-green or methyl-blue with eosin. Also, some intestinal canals were pulled out and fixed for later sectioning.

Thanks are due to Dr. L. R. Cleveland, who enabled me to obtain this material while collecting for him through a grant given to him by the Bache Fund of the National Academy of Sciences. I am also indebted to Dr. T. E. Snyder for identification of the termites and to Mr. J. E. Zetek, resident custodian of the Barro Colorado Island Biological Station, for assistance in collecting.[1]

[1] Most of this work was done at Yale University. Thanks are due to the University of Minnesota, where some facilities were generously given me during the summer of 1926.

II. The Intestinal Amoebae of Mirotermes hispaniolae Banks.

1. Endamoeba disparata sp. nov.

(Plates 22, 23 (figs. 17–26), and 24.)

General Morphology.

This amoeba, the most abundant species present in the intestine of its host, is intermediate in size between its two associates, from which it differs not only in this respect but also in its degree of activity, in the structure of the nucleus, and in the kind of cytoplasmic inclusions. The body may be spherical or more commonly ellipsoidal in form. While most of the fixed individuals possess blunt, short protuberances over much of the surface, there are sometimes a few large, clear, lobe-like pseudopodia. Although clear enough for facile observation of the nucleus, the endoplasm is usually crowded with small particles of wood and yellow, rounded objects of unknown nature.

The nucleus, which is generally situated toward one side of the animal, is spherical in what appears to be the resting stage (figs. 1–3, Pl. 22), but sometimes there is, as shown in some of these figures, a depression at one side, which is doubtless abnormal. While it ranges in diameter from 7·5 to 9 microns, it generally measures about 8.

The structure of the resting nucleus differs from that of nuclei in most other known entozoic amoebae. Situated near the nuclear membrane, against which they may be more or less flattened, there may be several nucleoli, usually from two to four but sometimes as many as seven, which are variable in size and shape. The largest of these is sometimes 2 microns in its longer diameter. These nucleoli stain with iron haematoxylin more heavily than does the chromatin. This latter substance is aggregated in an irregular granular mass which occupies most of the inner region of the nucleus and is surrounded by a clear zone, often of width one-third to one-half of the radius, which is apparently crossed by many radial filaments. This mass does not stain as heavily as do the chromo-

somes which arise later. In it the chromatin granules are small and often arranged more or less in lines. In the nucleus shown in fig. 4, Pl. 22, the chromatin granules are, as in the segmented spireme, arranged in threads, some of which extend out to the membrane.

It is remarkable that of two hundred and thirty-four individuals on seven slides, the nuclei of just half were in the form of a spindle (figs. 6, 7, 10, 11, 13, Pl. 22). This resembles a figure of mitosis, but its abundance makes it evident that it is a persistent stage. Although some spindle-shaped nuclei occur on all slides, the proportion is not always 50–50 ; on seven slides it varied from 72–28 to 27–66. The remaining small percentage of nuclei in the latter case were destroyed by parasites (see p. 196).

The average size of the amoebae with fusiform nuclei differs from that of those with spherical nuclei, the former being larger, but otherwise the structure of the two is similar. Measurements of two hundred and fifty individuals gave the following sizes, in microns :

Amoebae with spherical nuclei :

	Range.	*Range of Size Averages.*	*Average.*
Spherical body	20–39	22–28·5	27
Ellipsoidal body	13×21 to 25×39	19×28 to 26×35	24·5×31·5

Amoebae with fusiform nuclei :

Spherical body	20–41	26·5–35·5	33·5
Ellipsoidal body	16×27 to 34×52	25×32 to 33×42	29×38

In computing these averages it was found that on one slide all individuals were much smaller than usual, so, while these are included in the range of size, they were omitted in averaging. Some factor had produced in this termite smaller-sized amoebae than normally occur.

The fusiform nucleus is frequently about 14 microns long. One end is sometimes more pointed than the other, and when stained with Heidenhain's haematoxylin there is always a deeply staining substance at this pole (figs. 6, 7, Pl. 22), which usually has pushed out the membrane to a mammiform pro-

tuberance. Although with Delafield's haematoxylin this structure does not stain, it can still be distinguished (figs. 11, 13, Pl. 22). The peripheral nucleoli, which appear distinctly both when Heidenhain's and Delafield's are used, vary in number, size, and position, but are always more or less flattened against the membrane. The largest of these, which sometimes appears vacuolated, is beyond the equator toward the pole opposite to that characterized by the deeply stainable substance; others are in divers peripheral positions; and sometimes there is a small nucleus near or at the above-mentioned pole.

The chromatin is collected in a fusiform aggregate at the axis of the spindle, leaving a broad, clear zone between it and the membrane. This mass may be resolved into granules of various sizes (fig. 7, Pl. 22), but, as an end view shows, it is quite compact (fig. 8, Pl. 22). It is often uneven in outline and from it fibres run irregularly toward the nuclear membrane. Especially evident are two or three strands, along which may be some granules, which extend from the chromatin mass to the mammiform pole (figs. 13, 15, Pl. 22), but similar strands do not exist at the other pole. It is evident from the above descriptions that this spindle is not symmetrical, for the two ends differ morphologically in shape, in the mammiform protuberance at one, and in the strands at this same pole, as well as in the relative position of the peripheral nucleoli.

At first glance it seems possible that this unusual nucleus is undergoing a transitory stage of mitosis, but further consideration renders this doubtful in view of the absence on most of the slides of any stages other than this and the spherical form, and in view of the equality in numbers of these two types. Its form could be obtained from the more typical spherical nucleus by elongation, greater concentration of chromatin, and a suitable distribution of nucleoli. Thus it may follow this earlier phase of development, but why it should be so numerous I cannot suggest.

A few stages which appear to be transitional between these two forms were found. In them the nucleus (figs. 5, 12, Pl. 22) is more broadly ellipsoidal (in some more so than in those

figured) ; there may be several nucleoli against the membrane ; and the chromatin is concentrated into a less extensive, more compact mass, leaving a broad peripheral zone crossed by some strands. However, such stages are rare.

Mitotic Division.

Although karyokinetic figures were scarce in this material, I am able to give an account of this process from a study of about twenty figures from three slides, all of them stained in Delafield's haematoxylin without counterstain, which were very clear and easily interpreted.

In earlier stages the nucleus is spindle-shaped, the poles rounded, and the chromatin arranged in tangled threads on the central core. While in shape this nucleus is similar to the abundant spindles previously described, it differs in that it is symmetrical toward the two poles and possesses no nucleoli or other peripheral or polar structures. Possibly in a still earlier stage, before loss of the peripheral shape, the chromatin threads form, as in fig. 4, Pl. 22. In the mitotic spindles no centrioles can be seen, and no more darkly staining plasm at the poles, and the nucleoli have disappeared. The individual chromosomes, which are distinguishable at about this time, becomes more loosely arranged, occupying more of the intranuclear area (figs. 18–20, Pl. 23), and probably divide longitudinally, starting first at one end and before the final separation forming V's (figs. 18–20, Pl. 23). It will be noted that in this series there is no true equatorial plate stage ; intranuclear spindle-fibres are absent ; and the chromosomes have varicose outlines, as if composed of rows of granules. These characteristics, as well as the structure of the resting nucleus, distinguish Endamoeba disparata from intestinal amoebae of the genus Entamoeba, which have centrioles inside of the nucleus and small chromosomes which appear to be single granules.

In the anaphase (figs. 21, 22, Pl. 23), when the chromosomes have separated and converge to the poles, twelve can often be counted in each group. These are all varicose threads, and differ from one another in length. As the nucleus elongates and

constricts equatorially the poles become rounded (fig. 23, Pl. 23) until the dumb-bell form is attained, and then the middle constriction narrows to a thread which becomes longer than the original spindle before it ruptures (figs. 24–6, Pl. 23). No reorganization stages were observed.

During karyokinesis particles of food material crowd the endoplasm as during the vegetative stages.

Nuclear Parasitism.

The parasite Nucleophaga has been found destroying the nuclei in several species of both free living and parasitic amoebae. With its affinity for stains it contributes to some unusual nuclear appearances, which have sometimes been wrongly interpreted as phases of nuclear development. On the slides of Endamoeba disparata I observed numerous instances of this parasitism, some of which are shown on Pl. 24. Also on this plate is placed a figure of a normally nucleated amoeba (fig. 35, Pl. 24) for comparative purposes.

According to Dangeard (1895), the discoverer of Nucleophaga, its life-history is as follows. The parasite first appears within the nucleolus as a vacuole with a central granule, its nucleus—the clear cytoplasm of the invader appearing like a vacuole. The size increases along with multiplication of nuclei until the parasite occupies the entire interior of its host's nucleus and possesses more than a hundred of its own. At this time, when the parasite resembles a mulberry, sporulation to form zoospores begins. The sporangia reach maturity after the death of the amoeba. Doflein (1907, quoted by Chatton and Brodsky, 1909) observed copulation of motile, flagellated zoospores.

The youngest stages of the parasites of Endamoeba blattae observed by Mercier (1910) were small, irregular, granular masses with numerous nuclei. From an account of these his description passes to the formation of spores, small spherules which stain intensely with haematoxylin. These spores possess an investing membrane, and, within this, cytoplasm enclosing one or two chromatin grains. Mercier found

that after sporulation the membrane of the host's nucleus ruptures, permitting distribution of the spores in the cytoplasm of the amoeba.

A granular body in the nucleus of a small individual of Endamoeba disparata (fig. 37, Pl. 24) resembles the earliest stages of the parasite of Endamoeba blattae observed by Mercier. This, which exists along with the chromatin and nucleoli, can only be interpreted as an invading organism, for it resembles none of the usual nuclear structures. Most of the other Nucleophaga observed filled up the interior of the host's nucleus and possessed a large number of their own nuclei—several hundreds in the sporangia, for example, of figs. 41–3, Pl. 24. The spherules of these later stages stain intensely with iron haematoxylin and retain the stain so tenaciously that they remain coloured when all other structures have been decolourized. In some figures there are a few spherules of larger size (figs. 38, 39, Pl. 24), while in other, later stages these are more numerous and smaller (figs. 41–3, Pl. 24). The disintegrating chromatin is enclosed in the central zone and surrounded by several layers of spherules which come more and more to fill up the nucleus from the periphery inwards (fig. 40, Pl. 24). The diameter of the mature globe of spherules, which exceeds that of the original host's nucleus, may reach 12 microns.

When Delafield's haematoxylin or alum carmine is used the chromatin is coloured but the parasites remain mostly unstained, so that they may readily be distinguished from the components of the amoeba's nucleus and optical sections can be observed (figs. 42–3, Pl. 24) to show the arrangements described in the previous paragraph. By the use of these stains, as well as by a more complete differentiation of Heidenhain's haematoxylin, the spherules can be resolved into an investing envelope and a central chromatic granule (fig. 44, Pl. 24).

It is possible that figs. 36 and 46, Pl. 24, represent a different type of development of this Nucleophaga, if not a different species, as the structures drawn do not fit into the history of the form described above. The spherules of fig. 36, Pl. 24, are much too large to be nuclei in the latter series, and

the distributed spherules of fig. 46, Pl. 24, are also larger and less numerous than studies of the sporangia of this would seem to indicate the spores to be. That these spherules are parasites seems probable in the latter case because there is no nucleus present in the amoeba.

On most of these slides the parasites were present in an average of 8 per cent. of the nuclei, but this percentage varied from 6 to 12 on different slides and some seemed to be without any parasites.

Diagnosis and Summary.

Between 20 and 40 microns in longest diameter, averaging 32. Broad, blunt pseudopodia. Active in normal condition. Cytoplasm crowded with particles of wood and other material. Nucleus with distinct membrane, several peripheral nucleoli and an irregular granular mass of chromatin occupying a large part of the inner region; about 8 microns in diameter. In mitosis twelve varicose, filiform chromosomes formed from a spireme. Apparently no centrosomes or spindle-fibres and no definite equatorial plate. Xylophagous. Habitat: the intestine of Mirotermes hispaniolae Banks from the Canal Zone, Panama.

2. Endamoeba majestas sp. nov.

(Plate 25, figs. 47–53.)

The largest amoeba of the three species in the intestine of Mirotermes hispaniolae is present in considerable numbers, but is much less abundant than is Endamoeba disparata. The body, which may be spherical or broadly ellipsoidal with irregular outlines, ranged in fifty individuals on eight slides from 60×65 to 154×165 microns in size, averaging 94×115 microns. Many of the smallest animals occurred on one slide. There may be short, lobose pseudopodia protruded from various parts of the body (fig. 47, Pl. 25); a delicate but distinct periplast; and a thin, clear outer layer of cytoplasm which is conspicuous only in the pseudopodia. The endoplasm is densely granular and crowded with particles of wood in gastric

vacuoles ; some of the particles are very small, others relatively large. This food material, which is relatively more abundant than in Endamoeba disparata, stains deeply at times and obscures the nucleus, especially when iron haematoxylin is used.

The nucleus of this amoeba (figs. 48–51, Pl. 25) possesses a distinct but not especially heavy membrane. While its shape is typically that of a sphere, it is often deformed in preserved material. In diameter it ranges from 15–26, usually nearer 20 microns. The interior is occupied by an irregular reticulum which is more closely meshed toward the periphery and supports throughout some fine granules, which may be especially abundant at the outer portions. Near or at the centre is a dense aggregation of larger granules, sometimes very irregular in outline, which stains with chromatin dyes more or less deeply. The group is often quite dense and deeply staining, and presents then the appearance of a compact, granular endosome. In what seem to be early stages of karyokinetic organization these granules stain more deeply and take looser, linear arrangements, evidently forming thread-like chromosomes which become dispersed throughout the intranuclear area in the prophase, when the nucleoli, to be described below, disappear.

Irregular masses of nucleolar material are present in the region between the central chromatin aggregation and the membrane. Sometimes these are uneven, vacuolated masses which stain more intensely than does the chromatin when Delafield's haematoxylin is used—a circumstance also true of the nucleoli of Endamoeba disparata ; sometimes they are compact and rounded, or irregularly elongate ; and often they appear in winding linear shapes. As is apparent from the above description, the arrangement of nucleolar material is not the same in any two nuclei. The outer covering stains with chromatin dyes (Heidenhain's haematoxylin, Delafield's haematoxylin, and alum carmine) while the interior does not uniformly do so, with the result that the structures often appear vacuolated.

After suitable preparation, a relatively large granule, which is isolated by a surrounding clear area, may be seen at the centre of the chromatin mass (figs. 51–3, Pl. 25). Inasmuch as this

stains with iron haematoxylin more intensely than any other structure, it may be quite striking when the nucleus is very much destained. In appearance the granule is suggestive of the small karyosomes of Entamoeba and Dientamoeba, and possibly it is homologous to these.

Two amoebae were observed in which the nucleus contained a Nucleophaga similar to that of Endamoeba disparata.

In this species, as well as in the other amoebae of this termite, no cysts have been found in spite of much searching in many smears.

Diagnosis and Summary.

Between 65 and 165 microns in longest diameter, averaging 115 microns. Short, lobose pseudopodia often protruded from various parts of the body. Endoplasm crowded with food material, mostly fragments of wood. Nucleus between 15 and 26, usually nearer 20, microns in diameter. Interior of nucleus occupied by an irregular reticulum on which are many fine granules; a dense aggregation of chromatin granules near centre; a deeply staining granule in a clear area within this aggregation; and irregular, vacuolated masses of nucleolar material, often anguilliform, in outer zone. In division, the nucleoli disappear and the chromatin granules aggregate into threads. Xylophagous. Habitat: the intestine of Mirotermes hispaniolae Banks from the Canal Zone, Panama.

3. Endolimax termitis sp. nov.

(Plate 23, figs. 27–34.)

The smallest species of amoeba present in the intestine of its host, Mirotermes hispaniolae, seems to belong to the genus Endolimax. In shape the individuals preserved on the slides are, if definite at all, spherical, ovoidal, or ellipsoidal with irregular outlines, and there are usually no marked pseudopodial protuberances. The average size is $12{\cdot}5 \times 16$ microns, and the range between $9{\cdot}5 \times 9{\cdot}5$ or even smaller to 19×27 or rarely a little larger, as in the parasitized individual

of figure 34, Pl. 23. The cytoplasm is invested by a distinct pellicle which can be readily seen in instances when it is separated from the granular plasma, as frequently occurs, though in many cases at least this appears to be a result of the preservative treatments.

The cytoplasm is usually dark, granular, and more or less vacuolated. Within many of the vacuoles are small bodies which are doubtless ingested food materials, most probably small fragments of wood, and also in the endoplasm, surrounded by vacuoles, may be elongated fragments of wood or yellow, tubular structures similar to some of those observed in Endamoeba disparata. But the food material is never in abundance, so that the granular and rather dark endoplasm is more or less uniform in appearance, with no obtrusive crowding of large food bodies as in the relatively clear endoplasm of Endamoeba disparata.

The nucleus differs greatly in appearance from that of the latter amoeba. In form it is spherical; in diameter it varies in different individuals, though apparently not in ratio to the size of the body, from 4 to 5 microns. Occupying a large portion of the inner zone is an irregularly outlined karyosome, composed apparently of plastin in which are imbedded some granules. Besides these latter there sometimes appears to be a minute central granule (fig. 32, Pl. 23), set apart by a surrounding clear space. Peripheral chromatic granules cover the inside of the nuclear membrane, so that, as observed in optical section, a deeply staining granular ring forms the boundary. Extending across the clear zone between this peripheral chromatin and the karyosome there are numerous radial filaments.

In a prophase of mitosis the peripheral granules have disappeared from the nuclear membrane, the karyosome as such is no longer apparent, and the chromatic granules are arranged in a group of threads looped toward one side of the nucleus (fig. 33, Pl. 23). An important feature of this figure is the possible existence of an intradesmose with a granule at either end. But the type of mitosis remains unknown, for the only other division stage observed was a final stage in plasmotomy

in which the two nuclei were completely reorganized and the daughter individuals nearly separated.

Nucleophaga also parasitizes this amoeba, though apparently not very frequently in the specimens studied. Fig. 31, Pl. 23, seems to show a case in early parasitism in which there are only a few spherules within the nucleus, while the unusually large Endolimax termitis of fig. 34, Pl. 23, which has a cytoplasmic structure indicative of its belonging to this species, contains a large globe of spherules similar to the later stages in the development of the parasite of Endamoeba disparata.

In nuclear structure Endolimax termitis is similar to amoebae of the genus Endolimax, while it differs greatly from its associates in the termite.

Diagnosis and Summary.

Shape, when regular at all, spherical, ovoidal, or ellipsoidal; usually no definite pseudopodia; between 9·5 and 27 microns in longest diameter, averaging 16. Cytoplasm dark, granular; ingested particles of wood few. Nucleus 4–5 microns in diameter, with large central karyosome and many chromatic granules under the nuclear membrane. Xylophagous. Habitat: the intestine of Mirotermes hispaniolae Banks from the Canal Zone, Panama.

III. The Intestinal Amoebae of Mirotermes panamaensis Snyder.

1. Endamoeba simulans sp. nov.

(Plate 26, figs. 58–67.)

The larger of the two amoebae living in the intestine of Mirotermes panamaensis is also dominant in numbers. The body form is typically that of a sphere or ellipsoid, the amoeba is not very active, and when living it possesses many short, blunt pseudopodial protuberances over much of the surface (fig. 58, Pl. 26). A thin layer of clear ectoplasm can be distinguished in living and in suitably stained material, and

this is extensive in regions of pseudopodial formation, where it constitutes most of the broad, lobose extensions of the body sometimes present. Due to the large amount of ingested wood, which is, however, less abundant relatively than that of Endamoeba majestas, the amoeba is dark and the nucleus seldom can be easily seen. Fifty individuals on eight slides from three colonies ranged in size from 46 × 50 to 100 × 120 microns, averaging 65 × 77 microns, thus being smaller than Endamoeba majestas.

Though usually the nucleus is more or less obscured by the ingested fragments of wood, in some instances it appears clearly near the upper surface, and also some isolated nuclei may be found in the smears which have been released by rupture of the body, but nevertheless do not appear to be in any way abnormal. In diameter the nucleus ranges from 15 to 21, averaging 17·6 microns. It is invested by a definite but not unusually thick membrane, and the interior is differentiated into two areas (figs. 58–60, Pl. 26).

The outer of these two zones varies in width in what are apparently typical resting stages from a half to a third or even less of the radius, and appears quite clear except for alveolar walls and minute granules. It seems to consist of two morphologically distinguishable regions, an outer of many small alveoli and an inner of a single layer of large alveoli. The minute granules between the outer alveoli stain palely, so that the peripheral region is only slightly darkened. The walls of the larger inner alveoli appear like a series of radial fibres extending out from the sharply circumscribed inner mass.

While this outer zone is thus quite clear, the inner zone is occupied by an aggregation of chromatic material in which there are at least four morphologically distinguishable structures. At the periphery, in the first place, is a lightly staining, homogeneous or finely granular substance, perhaps plastin, which is probably the matrix in which the other materials are embedded and thus extends throughout the mass. In the second place, there are numerous lightly staining, moderate-sized granules distributed unevenly throughout the interior. At the inner

boundary of the outer plastin zone there are sometimes numerous granules of the same size which, however, take the stain intensely. The third structure is a large, deeply staining body near the central region, which body varies greatly in form, may appear as a group of irregular masses, and often appears vacuolated or unevenly granular. Finally there is a variable number of small spheres, apparently nucleoli, situated peripherally just within the plastin boundary. When the stain is intense these may be uniformly coloured, but often only the outer shell stains so that the nucleoli appear in optical section as rings, which are, however, not perfectly outlined. While sometimes these take the stain as intensely as does the chromatin, in other cases they are pale and inconspicuous while the chromatin is deep-coloured.

Nuclei of different appearance are frequently found (figs. 62–5, Pl. 26); these are apparently undergoing karyokinetic changes. Early in this process the central aggregation expands to the membrane, so that then the outer zone is absent. The nucleoli, now situated toward the periphery of the nucleus, become pale and finally disappear. Also the granules disappear, perhaps entering into construction of the chromosomes. The chromatic body becomes more regular in outline and more evenly granular, so as to appear as a karyosome. Varicose or granular chromatic threads form, at first centrally, then more dispersed in the intranuclear region. These may number about a dozen, and there is some evidence that they undergo the early longitudinal split characteristic of many chromosomes. No later stages of mitotic division in the trophozoite were observed, so the fate of these chromosome-like structures could not be traced.

Although, in spite of extensive observations on a large amount of material, no cysts were found in any other species described in this paper, several good examples were seen in *Endamoeba simulans*. However, even here, judging from specimens on smears, encystment is not common. Of those observed one possessed a single dividing nucleus, one two nuclei and another four, and the cytoplasm of all these was free of food particles.

An early stage of cyst development was exhibited by a rounded amoeba, measuring 65 × 72 microns, which seemed to be investing itself with a gelatinous covering. From the body all food particles had been extruded, but the cytoplasm contained numerous chromatin granules as well as the single nucleus. The form of this nucleus was that of a spindle with broadly rounded ends ; the internal structure was of an alveolar nature with a few chromatic granules unevenly distributed in it. Evidently it was in a phase of karyokinesis, but unfortunately the finer details of structure could not be accurately seen.

Each of the other two cysts, which are figured, possesses a thick hyaline wall, which in that with two nuclei is separated from the enclosed amoeba (fig. 66, Pl. 26). These have a uniform thickness of about 2·5 microns, and were covered by adherent debris. The transparency of the quadrinucleate cyst (fig. 67, Pl. 26) permitted a very clear view of the organism. Except for the complete absence of food particles, the cytoplasmic structure is similar to that of the trophozoite. Adjacent to the inner surface of the cyst wall is the investing periplast. The nuclei measure about 10 microns in longest diameter and contain numerous chromatic granules and aggregations of granules in a finely alveolar matrix. The quadrinucleate cyst measures 63 × 56 microns, the binucleate 60 × 72 microns. It is obvious from the appearance of these nuclei that the structure and type of mitosis in the nuclei of the encysted amoebae is, as in Endamoeba blattae, different from that of the trophozoites.

In the cytoplasm of this last encysted amoeba a number of granular aggregations are seen, and similar structures were seen in some of the trophozoites. Apparently these are cytoplasmic parasites, perhaps Sphaerita, which is not uncommon in the flagellates of termites as well as in many other Protozoa.

Also, as in at least two of the other species, the nucleus may be destroyed by Nucleophaga (fig. 61, Pl. 26).

Diagnosis and Summary.

Between 50 and 150 microns in longest diameter, averaging 77. Many short, blunt pseudopodia may be protruded from

various parts of the body. Endoplasm contains ingested wood, but less than does Endamoeba majestas. Nucleus 15–21, averaging 17·6 microns in diameter; two intranuclear zones, outer clear, inner dark. Chromatin in an irregular, karyosome-like mass in inner zone; small, spherical nucleoli at the periphery of this zone. In mitosis inner zone expands, nucleoli disappear, chromatin granules form threads which become dispersed within the nucleus. Xylophagous. Habitat: the intestine of Mirotermes panamaensis Snyder from the Canal Zone, Panama.

2. Endamoeba sabulosa sp. nov.

(Plate 25, figs. 54–7.)

A smaller amoeba is also present in the intestine of Mirotermes panamaensis. From a few termites of this species which were dissected in Panama I recorded many small amoebae which moved actively, were quite clear, and were comparable in numbers and general appearance to Endamoeba disparata of Mirotermes hispaniolae. But while in some ways this amoeba described below resembles this latter species, it possesses several specific differences.

The body of the amoeba is broadly ellipsoidal, rarely spherical in shape, and generally has no pseudopodial protuberances (fig. 55, Pl. 25). In some individuals, however, a few short, broad pseudopodia have been preserved (fig. 56, Pl. 25). Fifty individuals on nine slides averaged 22×26 microns, ranging from 15×19 to 32×35, but the extremes are exceptional. Most individuals measure close to the average size.

Particles of ingested wood are present in the cytoplasm, but much less abundantly than in Endamoeba disparata. These are generally small, but are sometimes of relatively large size. The most characteristic feature is the large number of chromatic cytoplasmic granules (figs. 54–7, Pl. 25), which in size and form resemble the smaller mitochondria of Endamoeba gingivalis (Causey, 1925). These appear in all individuals in proportionately similar numbers, whether the

material was fixed in Schaudinn's or Bouin's fluids ; they stain intensely with iron haematoxylin but not with Delafield's haematoxylin ; and they are often especially numerous against and near the nucleus. These inclusions are evidently metabolic products, but differ from mitochondria in their behaviour toward reagents, for they are easily demonstrated in material fixed by ordinary methods. A number of smaller, non-staining, refractile, cytoplasmic granules are also present in all preparations.

The nucleus is generally spherical, 7–10 microns in diameter, and resembles in size and structure that of Endamoeba disparata (fig. 55, Pl. 25). It agrees with that of the latter species in the possession of a delicate membrane, an irregular, granular mass of chromatin nearly filling the interior, and several irregular, deeply staining nucleolar bodies peripheral to this mass. Of these last there are usually not more than four. The aggregation of chromatin granules varies in extent from not more than half the diameter of the nucleus, in which case there is a broad clear zone surrounding it, to a nearly complete occupancy of the intranuclear region. The latter condition is more common. In that the nucleolar bodies are always against this mass, never just under the nuclear membrane, the arrangement differs from that of Endamoeba disparata.

Occasionally a different type of nucleus (fig. 56, Pl. 25) may be found in individuals which otherwise resemble the usual form. This has a concentrated central mass, about half or less of the nuclear diameter in extent, surrounded by a broad zone which is clear except for indefinite lines and granules. On its periphery are several (commonly three or four) rounded nucleolar bodies, in the centre is often apparent a relatively large karyosome-like structure, and the substance is indistinctly granular and lightly staining. Sometimes (fig. 57, Pl. 25) there are two nuclei of this type applied to one another so closely that the adjacent parts are flattened. It is noteworthy in this connexion that although as many as six of the amoebae with pairs of nuclei appeared on one slide, and several on others, no binucleate amoebae with separated nuclei were found. The somewhat smaller size of the amoebae in which such nuclei occur suggests

that it may follow division. The arrangement is similar to that in Sappinia diploidea (Hartmann and Nägler, 1908).

Two poorly preserved late anaphases showed that in this amoeba there are no filiform chromosomes such as those of Endamoeba disparata, but instead small, concentrated masses of chromatin at the poles.

Diagnosis and Summary.

From 19 to 35 microns in longest diameter, averaging 26. Ingested food material not abundant. Endoplasm contains a large number of granules which stain deeply with iron haematoxylin and resemble some mitochondria in size and form; these may be especially abundant about the nucleus. Nucleus 7–10 microns in diameter, irregular mass of granular chromatin occupies most of the central zone, several rounded nucleoli at periphery of this. In some individuals chromatin is more concentrated in nuclei which may be single or, less commonly, in couples closely applied to one another. Xylophagous. Habitat: the intestine of Mirotermes panamaensis Snyder from the Canal Zone, Panama.

IV. Discussion.

From the point of view of the student of Protozoa and of those who are concerned with the host-parasite relationships in termites, these amoebae possess several features of interest. First of all they are noteworthy because of their peculiar nuclear structures and xylophagous habits; in the former of which four, in the latter all of the species differ from other entozoic amoebae which have been described, whether from invertebrates or from higher animals. Apart from brief notes concerning these forms given by myself (1925, 1926), Cleveland (1926), and Snyder (1926), I am not aware of any published records of amoebae from termites; but before I obtained this material Light had observed a small, apparently non-xylophagous species in Kalotermes simplicicornis. In the second place, at least one of the two largest amoeba has some characteristics which indicate a relationship to Endamoeba blattae, which has

hitherto been isolated.[1] Furthermore, several questions suggest themselves concerning the relation between these Protozoa and their hosts, and although these cannot be answered at present for lack of complete knowledge and experimental data, several probabilities may be outlined. Do these amoebae share in the symbiotic relationship which has been demonstrated to exist between lower termites and their intestinal flagellates? Are they simply commensals, or do they assist the host in the digestion of wood? Whence did Mirotermes and the other Termitidae obtain their infections? The small intestinal amoebae from four other infected species of the Termitidae will be described later.

As few cysts have been seen, and these in only one species, and as the mitotic phenomena are well known in only one case, the typical nuclear structure is the only character which can be used to advantage for comparative purposes. Each of the five has a characteristic nuclear structure which differs from that of the others. Endamoeba disparata and Endamoeba sabulosa are obviously related, Endamoeba majestas and Endamoeba simulans less obviously so both with each other and with the previously mentioned species, and Endolimax termitis stands alone in this group.

The nucleus of the last-mentioned species, having a large, compact karyosome like that of the genus Endolimax, closely resembles the nucleus of Endolimax ranarum (Epstein and Ilovaisky, 1914) or the 'limax amoeba from Tipula, Vahlkampfia sp.?' recorded by Mackinnon (1914)

[1] Since writing this I have observed in the intestine of Periplaneta americana, together with typical Endamoeba blattae, amoebae very similar to Endamoeba disparata. These are numerous in some cockroaches, they are fairly uniform in size, and the structure of the nucleus is very much like that of the termite's amoeba. They also agree with the latter in size. At first sight these seem to constitute a distinct species, although apparently they have hitherto been regarded as stages in the life-history of Endamoeba blattae. These, of course, afford more evidence for the common origin of the amoebae of Mirotermes and those of the cockroach, whether or not they are distinct from Endamoeba blattae.

and others. If the genus Endolimax is accepted, Endolimax termitis probably belongs there. Boeck and Stiles (1923) and Craig (1926) suggest that the genus is doubtful, and should perhaps be a synonym or sub-genus of Endamoeba.

The four species which have been placed in the genus Endamoeba (with the type Endamoeba blattae, not corresponding to that of Boeck and Stiles) seem to be more closely related to the type species of that genus than to entozoic amoebae of other well-established genera. Two of them resemble in nuclear structure Branchipocola (Breindl, 1925) more than others, but not sufficiently to warrant an assignment to that genus, established for an amoeba from the crustacean Branchipus grubyi. The alternative would be to give them distinct generic names, but it does not seem wise to multiply genera for these closely associated forms. If the definition of the genus Endamoeba can be extended to include such forms, perhaps they should at least be given sub-generic distinction.

English workers have pointed out the error in confusing the genus Entamoeba of Casagrandi and Barbagallo (type: Entamoeba coli) with the genus Endamoeba of Leidy (type: Endamoeba blattae). Because these type species are generally different, Endamoeba stands for Endamoeba blattae and cannot be used for the others, so that the entozoic amoebae of the coli type must be given another name. Perhaps the use of Entamoeba is not justified, in view of its similarity to the earlier name; then it must be something else (as Poneramoeba Lühe).[1]

Several amoebae of the Entamoeba coli type have been found in insects; as Löschia hartmanni Mackinnon (1914) from larvae of crane flies; Entamoeba belostomae

[1] The International Commission on Zoological Nomenclature has recently (1927, 'Smithsonian Miscellaneous Collections', vol. 73, no. 4, p. 14) placed in the Official List of Generic Names Endamoeba Leidy, 1879 *a* with type blattae Bütschli, 1878 *a* from Blatta orientalis. While this action officially designates Endamoeba as the generic name of its type, it does not settle the controversy concerning the entozoic amoebae which are not congeneric with this.

Brug (1922) from the water bug; Entamoeba apis Fantham and Porter (1911) from the bee; Entamoeba sp. from the cockroach, Lucas.

Since Endamoeba blattae has an important place in this discussion, I shall give a brief description of it from published accounts. Among those recently published, Wenyon's summary of the morphology and life-history is probably available to all interested; Kudo (1926) has given a description of division; and Thomas and Lucas (1926) have published a brief account corroborating Mercier (1910). Inasmuch as Endamoeba blattae is a comparatively well-known species, I shall limit this sketch chiefly to a comparison of the occurrence, size, nuclear structure, and division with the same phenomena in Endamoeba majestas and Endamoeba simulans.

Records of infection of cockroaches with Endamoeba blattae vary from 4 to 50 per cent. This incidence of infection differs from that of the amoebae from termites, which seem to occur in all individuals—a result of the perfect transmission by proctodaeal feeding.

Since reproduction by cysts plays only a minor part in the life cycle of these amoebae of termites, if, indeed, cysts occur at all in Endamoeba majestas, the variation in size is not so great as in Endamoeba blattae. A comparison of size should therefore take into consideration only the larger vegetative individuals of Endamoeba blattae, for those hatched from cysts are, of course, much smaller than they later grow to be. These larger individuals are smaller than Endamoeba majestas, but are comparable in size to Endamoeba simulans, averaging above 50 microns and ranging up to 130 microns. The diameter of the nucleus is also similar to that of this latter species.

The nuclear membrane of Endamoeba blattae is remarkably thick, much thicker than in our forms. Within this two zones may be distinguished, the outer dark and the inner clear. In life the outer zone contains numerous refractile granules which dissolve on fixation and expose a finely reticular structure on which are numerous chromatin granules. Whether

or not these refractile granules exist in the termite amoebae is unknown, for I was unable to study the living nucleus with these in mind. The inner zone is coarsely reticulated or alveolar, free from chromatin granules, and otherwise, according to Kudo (1926), shows no particular structure. In Janicki's previous work (1909) chromatin granules were found in the inner zone, and a large karyosome, according to Kudo the ' separated central reticulum ', was described, but these observations have been contradicted by later workers. Between these two zones occur numerous nucleoli similar in form and arrangement to those of Endamoeba simulans. Wenyon states that these are large chromatin granules, but Janicki, Mercier, and Kudo regard them as nucleoli, as I have done. In probably degenerating nuclei Kudo finds that these nucleoli may fuse together in threads or masses, when they resemble those of Endamoeba majestas. In the latter, however, as regards the form of the nucleoli, there is no question of degeneration.

However, the two zones in the nucleus of Endamoeba simulans are not comparable with those of Endamoeba blattae, for in the former the outer zone is clear and the inner, darker zone contains the chromatin, mostly in an irregular karyosome-like structure. The most striking resemblance is in the arrangement and form of the nucleoli. Endamoeba majestas differs still more, resembling the type only in the arrangement of differently formed nucleoli, a form which may sometimes appear in Endamoeba blattae.

After the structure of the resting nucleus, we have next to consider the changes taking place during karyokinesis. As in the amoebae from termites the nucleoli of Endamoeba blattae disappear early in this process and their place is taken by fine chromatin granulations, which I do not believe to be related to the former structures. Chromatin granules then appear in the central reticulum, enlarge and organize into a thread which later breaks into a number of segments. This course of events is very similar to that of Endamoeba simulans and also of Endamoeba majestas, where the nucleoli disappear and filiform chromatic bodies appear in the

central region. Mercier (1910) calls these pseudochromosomes, while Kudo (1926) finds them to be linear groups of chromatin granules. In Endamoeba blattae these become arranged into two groups and move toward the poles, and apparently no centrosomes or spindle structures have been found in the vegetative amoebae since the doubtful observations of Janicki.

Furthermore, the cysts of Endamoeba simulans somewhat resemble those of Endamoeba blattae. Those of the latter measure from 30 to 50 microns in diameter and possess thick, hyaline walls; while those of the former, with similar walls, are somewhat larger. Since only three cysts of Endamoeba simulans were found, little is known of the number of nuclei, but morphologically these seem to resemble those of the other species in the irregular distribution of a limited number of very large chromatin granules on a reticular substratum, and sometimes a denser aggregation perhaps comparable to what Mercier interprets as a karyosome containing a centriole.

Inasmuch as Endamoeba simulans and Endamoeba blattae, besides resembling each other in size and encystment, possess certain nuclear features in common, it seems defensible to conclude that the two are related, though possibly they differ sufficiently to warrant sub-generic distinction. While Endamoeba majestas, with the small central karyosome surrounded by a dense aggregation of chromatin granules and a clear alveolar peripheral zone in which are situated the elongate nucleoli, shows few similarities during the resting stage, it shows some during organization of the nuclear material prior to mitosis.

Endamoeba disparata can be more definitely placed, for its manner of nuclear division is known. Both in the structures of the resting and dividing nucleus it stands apart from most intestinal amoebae hitherto described. It is evident that in its peripheral nucleoli and large, granular mass of chromatin, with no karyosome; in the curious spindle form of the nucleus which is as prevalent as the other type; and in the formation of filiform chromosomes during division it is unique among intes-

tinal amoebae. In this last point, it seems to accord in some degree with the type species of the genus. It also differs from many other entozoic amoebae in the absence of intranuclear centrosomes and spindle-fibres. The type of division makes an interesting addition to the cytology of this group of Protozoa.

It has already been stated that in the structure of the resting nucleus, as well as in some general characters of the body, Endamoeba sabulosa shows some affinities to Endamoeba disparata, but in the absence of knowledge of the nuclear division, apart from two fragmentary observations of telophases which seem to show some differences from the latter species, the closeness of this relationship is uncertain. The significance of the other type of nucleus in this species, and of the peculiar Sappinia-like binucleate condition, is at present unknown.

One would expect to find cysts, since they are the propagative stages of most entozoic amoebae. Such intestinal amoebae as Endamoeba blattae and Entamoeba coli could not often, if ever, infect new hosts without the aid of cysts. But, when a more direct method of infection is provided, as in the case of Endamoeba gingivalis, cysts may not be formed. The habit of termites in taking proctodaeal food directly from one another makes the transmission of active Protozoa simple, so that encystment may not always take place. However, although no cysts were seen on these slides except in the case of Endamoeba simulans, it is not certain that there are none in the life-histories of the other amoebae, for, owing to the death of the amoebae in the transported termites, careful investigation of faecal deposits was not possible.[1]

Finally, the parasites of nucleus and cytoplasm may be mentioned. Nucleophaga infests the nuclei of four species: Endamoeba disparata, Endamoeba majestas, Endamoeba simulans, and Endolimax termitis,

[1] Living termites were sent to Dr. Cleveland, but most of the amoebae died during transportation and the termites did not survive for more than a few months.

most commonly in the first ; and Sphaerita is not uncommon in the cytoplasm of Endamoeba simulans.

There remains for consideration the problem of the relation of these amoebae to their hosts. This has already been briefly discussed by Cleveland (1926) and Snyder (1926). Although abundant, the amoebae do not occur in the closely packed condition usual among the flagellates in the Kalotermitidae and Rhinotermitidae, where a section through the intestine will show it to be completely filled with Protozoa. Because of the small numbers, it does not seem probable that they could prepare all the food used by the host, as is supposed to be the case with the true symbionts. Also, Mirotermes will live at least for many weeks without the amoebae. On the other hand, certain considerations render it very possible that the amoebae may be at least of some service to the host for the same reason which gives the flagellates this credit. The wood on which this termite feeds seems to be in the same condition as that eaten by lower termites, and probably is acted upon by the amoebae in the same manner as it is by the symbiotic flagellates.

Infection may have been carried on for ages from individual to individual and colony to colony. But whence did it originally come ? Was it obtained during development from lower termites most of the nearer relatives of which have since lost their amoebae, for, as already stated, none of those examined, except Kalotermes simplicicornis, contain any ; or has it been acquired later by the Termitidae, which have lost the large flagellates ? The possibility of obtaining infection from other coexisting species of termites seems remote, because of the isolated habits of termite colonies ; and also improbable, for the same reason, seems the possibility of infection by cysts from other animals. If there is any real affinity between Endamoeba simulans and the others and Endamoeba blattae, as well as between Endamoeba disparata and the smaller amoeba of the cockroach, the line of development must lead to the ancestors common both to the termite and cockroach.

In conclusion it may be well to reiterate what Snyder (1926)

has already mentioned ; that is, that the statements sometimes encountered that the Termitidae contain no Protozoa are unwarranted. It is true that at least most of those examined contain no faunas comparable to those of other families, but these records from Mirotermes evince the possibility of abundant infections being found in some of the Termitidae which are yet to be studied. Others may contain small amoebae and flagellates which are few in number, inconspicuous and difficult to study, but nevertheless they are of considerable interest.

V. Appendix.

Gregarina mirotermitis sp. nov.

For the sake of completeness, a brief note concerning a gregarine found in Mirotermes panamaensis may be given. Gregarines have been found infrequently in the intestines of certain termites. Leidy (1881) first recorded Gregarina termitis from Reticulitermes flavipes of the eastern United States : Ellis (1913) observed a larger form in western termites, probably Reticulitermes hesperus, in which I have often seen them ; they are sometimes abundant in Termopsis ; and Bequaert (1925) mentions having found a gregarinid ' apparently of the genus Stylocephalus (Stylorhynchus) ' lining the intestinal wall of Neotermes castaneus in Brazil. This last was ' provided with a long, snout-like appendage or epimerite, fixed in the epithelium '. It seems possible, however, that this was Oxymonas, which I have found in Neotermes holmgreni as well as in all other members of the sub-family Kalotermitidae which I have examined. Oxymonas, which frequently adheres in large numbers to the intestinal wall by means of the long ' proboscis ', superficially resembles in general form some stages in the life-history of Stylocephalus.

To these records I may add a description of the gregarine found on slides prepared from Mirotermes panamaensis. It obviously differs from Gregarina termitis, but in the absence of gametocysts or any stages other than the free gre-

garines, it is impossible to define it completely. However, it is undoubtedly a new species, and may be named Gregarina mirotermitis.

Each of the two pairs observed consisted of two individuals in syzygy. One of these is figured on fig. 68, Pl. 26. In this the primite is larger than the satellite, which is attached to it by a deep cup in the protomerite receiving the narrowed posterior end of the anterior individual. Measurements from fixed material will be of value, though they cannot be accurate. The total length of the primite is 94 microns, of which the deutomerite is 79 ; the width of the protomerite is 16, of the deutomerite 39. The total length of the satellite is 65 microns, of which the deutomerite is 59 ; the width of the protomerite is 14, of the deutomerite 27. Thus this gregarine may be of about the same length as Gregarina termitis (60 microns), or may be larger, but it is proportionately narrower and the protomerite is relatively much smaller. The septa are distinct, and the surface of the body is delicately striated longitudinally. Host : Mirotermes panamaensis Snyder from the Canal Zone, Panama.

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DESCRIPTION OF PLATES 22–6.

All figures were drawn with a 2 mm. oil-immersion objective, oculars 10 or 20, and a camera lucida at magnification 1,440 or 2,740. The material on Pls. 22, 23, 24 was fixed in Schaudinn's fluid ; the rest in Schaudinn's or Bouin's as stated. The staining of each figure is indicated by abbreviation in the legend, as follows : H., Heidenhain's iron haematoxylin ; D., Delafield's haematoxylin ; AC., alum carmine ; M., methyl-blue and eosin.

Plate 22.

Figs. 1–16. Endamoeba disparata sp. nov. from Mirotermes hispaniolae Banks.

×2,000. All figures may be measured by the scale of microns.

Figs. 1–2.—Entire amoebae showing food particles and resting nuclei with peripheral nucleoli and granular chromatin mass. The nuclei are somewhat deformed. H.

Fig. 3.—Resting nucleus showing linear arrangement of granules. D.

Fig. 4.—Nucleus showing arrangement of granules in definite threads. H.

Fig. 5.—An enlarged nucleus in entire amoeba showing concentration of chromatin mass. D.

Figs. 6–7.—Spindle-formed nuclei, which occur in half the amoebae. Note mammiform protuberance at one pole and granular chromatin mass. H.

Fig. 8.—An optical transverse section of a fusiform nucleus. D.

Fig. 9.—Fusiform nucleus in entire amoeba. Dense nucleolar substance, which always appears in iron-haematoxylin material, at one pole. H.

Figs. 10, 11, 13.—Spindle-formed nuclei stained in Delafield's. Note the different staining of the polar nucleoli.

Fig. 12.—Nucleus showing concentration of chromatin mass, flattening of nucleolus, and enlargement. Possibly transitional to spindle shape. Compare with fig. 5. D.

Fig. 14.—Chromatin granules arranged in lines in fusiform nucleus. D.

Fig. 15.—An unusually large spindle-formed nucleus, with a large, flattened nucleolus. D.

Fig. 16.—A smaller amoeba. A.C.

Plate 23.

Figs. 17–26. Endamoeba disparata sp. nov. Karyokinesis.

Figs. 27–34. Endolimax termitis sp. nov. from Mirotermes hispaniolae Banks.

×2,000. All figures may be measured by the scale of microns.

Fig. 17.—Spindle nucleus in prophase; chromosomes arranged in close mass which possibly follows fig. 14, Pl. 22. D.

Fig. 18.—End view of an early prophase stage in which there is some evidence of a V-like separation of chromosomes. D.

Fig. 19.—Lateral view of a similar stage, also some evidence of V-like separation of chromosomes. D.

Fig. 20.—Chromosomes loosely arranged in late prophase. D.

Fig. 21.—Entire amoeba, nucleus in anaphase. Food particles in body. D.

Fig. 22.—Anaphase. There are twelve chromosomes in each group. D.

Fig. 23.—A later anaphase in which the poles are becoming rounded. D.

Figs. 24–6.—Telophases showing elongation and disappearance of the strand connecting the two nuclei. Twelve chromosomes may be counted in each of these. D.

Fig. 27.—Endolimax termitis showing vacuolization of cytoplasm, karyosome, peripheral chromatin, and radial filaments in nucleus. D.

Fig. 28.—A similar stage, differently formed body. D.

Fig. 29.—Fewer peripheral granules. A.C.

Fig. 30.—Granules in karyosome, as also indicated in previous figures. D.

Fig. 31.—Possibly early stage of Nucleophaga in nucleus. H.

Fig. 32.—Little cytoplasm surrounding nucleus, which shows granulation of karyosome and central granule. H.

Fig. 33.—A stage of early organization for division. Chromatin threads and possibly intradesmose. D.

Fig. 34.—A large sporulation body of Nucleophaga. D.

Plate 24.

Figs. 35–46. Endamoeba disparata sp. nov. Nucleophaga. ×2,000. All figures may be measured by the scale of microns.

Fig. 35.—A normal amoeba with the usual form of nucleus, inserted here for comparison with parasitized nuclei. H.

Fig. 36.—Large spheres in nucleus. Early in development of Nucleophaga, possibly a different form from most others on this plate. H.

Fig. 37.—An early stage in parasitism by Nucleophaga. Spherical, granular mass in nucleus. H.

Fig. 38.—Sporulation of Nucleophaga. A large mass of deeply staining spherules. H.

Fig. 39.—A similar stage in entire amoeba. H.

Fig. 40.—An optical section of a parasitized nucleus showing degenerating chromatin in centre. H.

Fig. 41.—Surface view of a similar stage. H.

Figs. 42–3.—Similar stages stained in Delafield's showing the pale stain of Nucleophaga.

Fig. 44.—A mass of developing spores showing chromatic granules within an envelope. H. Counterstain acid fuchsin.

Fig. 45.—Looser, more irregular arrangement of spherules. The amoeba is ingesting a piece of wood. H.

Fig. 46.—Thirty-eight large spherules which have perhaps ruptured the nucleus, for none is present. D.

Plate 25.

Figs. 47–53. Endamoeba majestas sp. nov. from Mirotermes hispaniolae Banks.

Figs. 54–7. Endamoeba sabulosa sp. nov. from Mirotermes panamaensis Snyder.

Magnification as stated. Fixation of figs. 54–7 Bouin's, others Schaudinn's.

Fig. 47.—Entire individual showing body-form and wood. D. ×1,056.

Figs. 48–50.—Nuclei showing granulated reticulum, central mass of chromatin, anguilliform nucleoli, karyosome in fig. 50. A.C. ×2,000.

Fig. 51.—Central karyosome and irregular nucleoli. H. ×2,000.

Fig. 52.—Entire individual containing large pieces of wood. Note that this is much larger than the individual of fig. 47. H. ×575.

Fig. 53.—A single nucleus showing irregular nucleoli, karyosome, and granular chromatin. H. ×1,056.

Fig. 54.—A large individual of Endamoeba sabulosa. Note nucleus, small amount of wood in body, many mitochondria-like granules. B.H. ×2,000.

Fig. 55.—A smaller individual, similar structure. B.H. ×2,000.

Fig. 56.—A different form of nucleus, more concentrated. B.H. ×2,000.

Fig. 57.—A binucleate individual, with the nuclei closely applied to one another. This is not a cyst. B.H. ×2,000.

Plate 26.

Figs. 58–67. Endamoeba simulans sp. nov. from Mirotermes panamaensis Snyder.

Fig. 68. Gregarina mirotermitis sp. nov. from Mirotermes panamaensis Snyder.

Magnification, fixation, and staining as stated. All figures except those of individual nuclei may be measured by scale.

Fig. 58.—Entire individual showing pseudopodial protuberances and nucleus in resting condition. B.H. ×1,056.

Figs. 59–60.—Isolated nuclei showing two zones, nucleoli, granular karyosome-like mass of chromatin, &c. B.H. ×2,000.

Fig. 61.—Nucleus parasitized by Nucleophaga. S.M. ×1,056.

Fig. 62.—Outer zone suppressed, nucleoli pale, chromatin in threads. B.H. ×2,000.

Fig. 63.—A similar stage to previous one; the threads seem to be splitting. S.H. ×2,000.

Fig. 64.—A similar stage to fig. 62. Nucleoli seem to be absent. B.H. ×2,000.

Fig. 65.—A large individual, chromatin threads formed in nucleus. B.H. ×1,056.

Fig. 66.—A binucleate cyst. B.H. ×1,056.

Fig. 67.—A quadrinucleate cyst. B.H. ×1,056.

Fig. 68.—*Gregarina mirotermitis*. Two individuals in syzygy. B.H. ×1,056.

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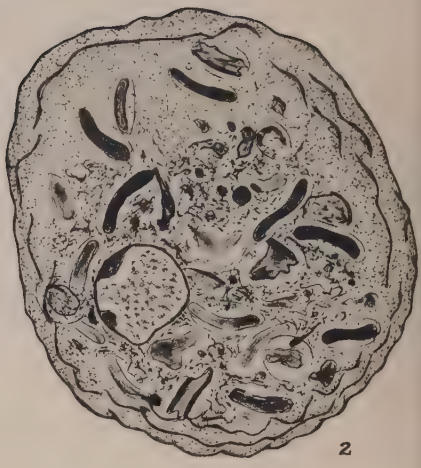

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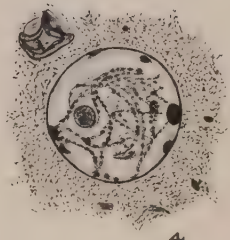

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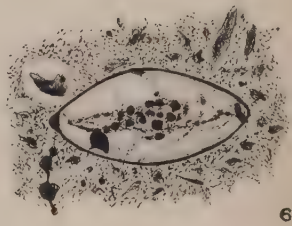

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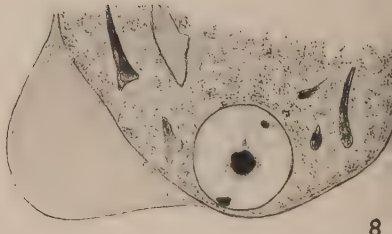

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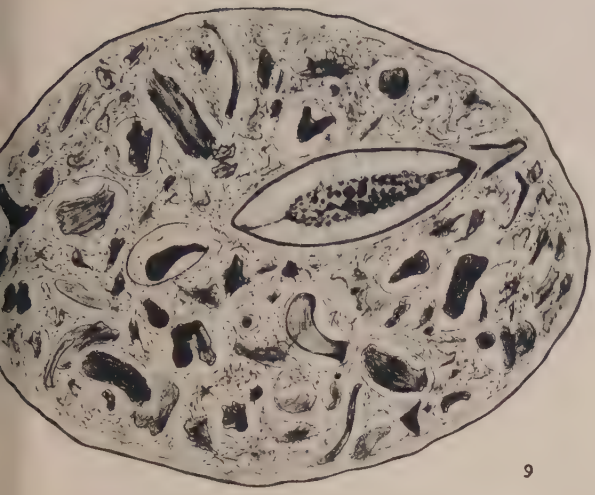

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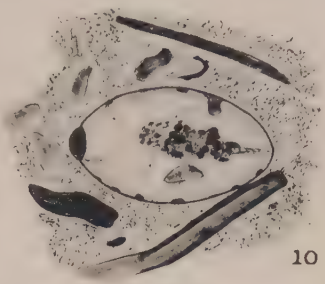

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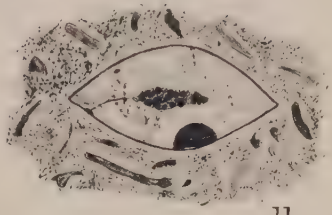

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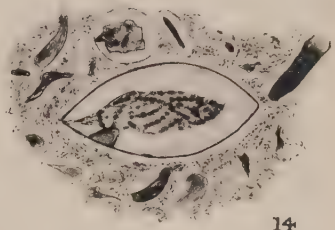

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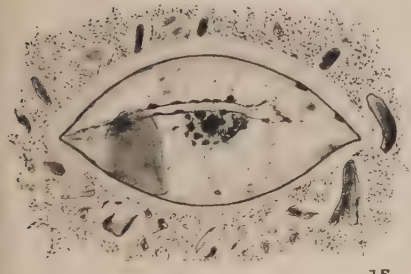

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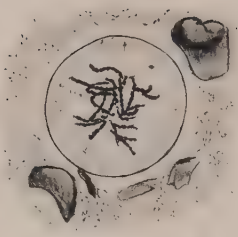

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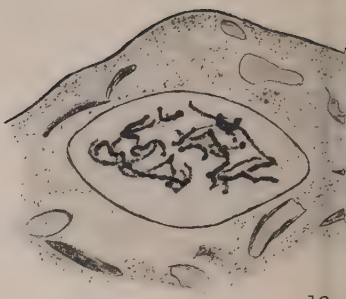

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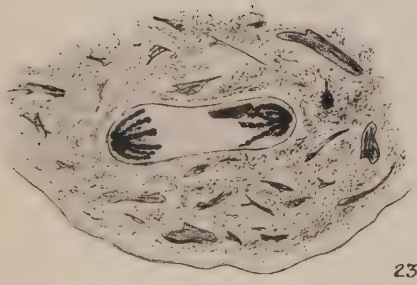

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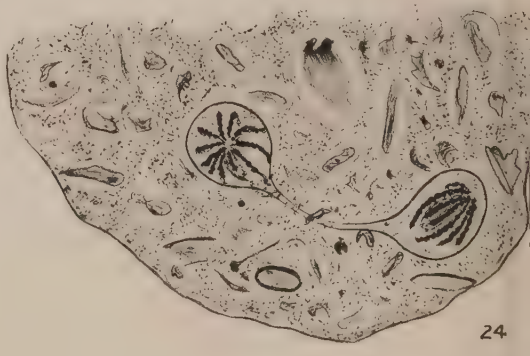

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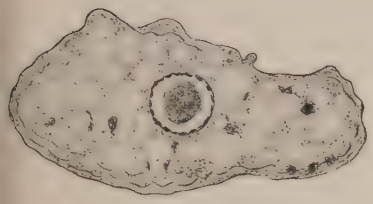

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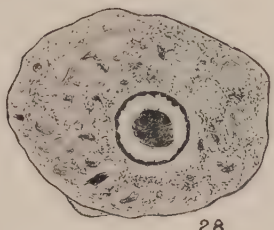

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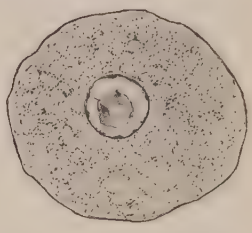

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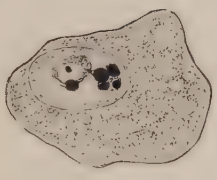

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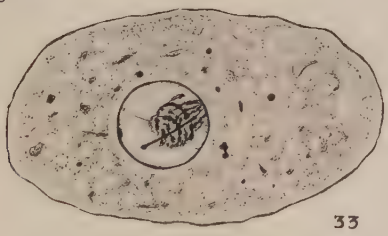

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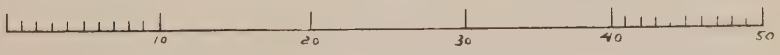

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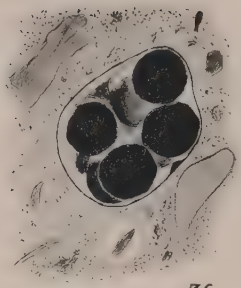

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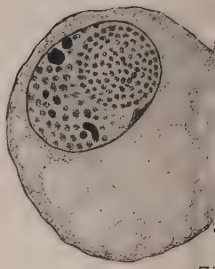

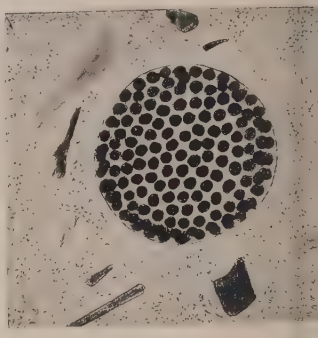

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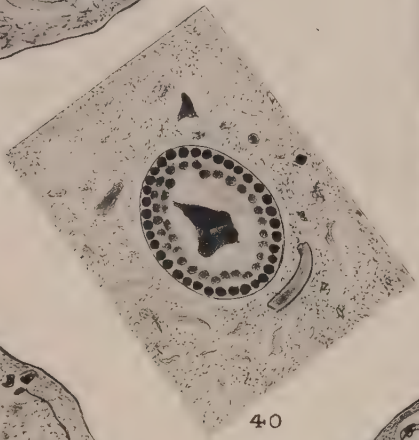

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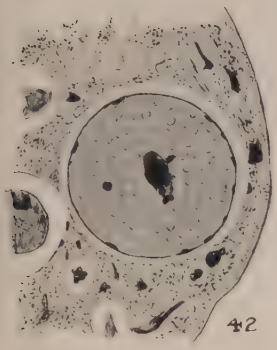

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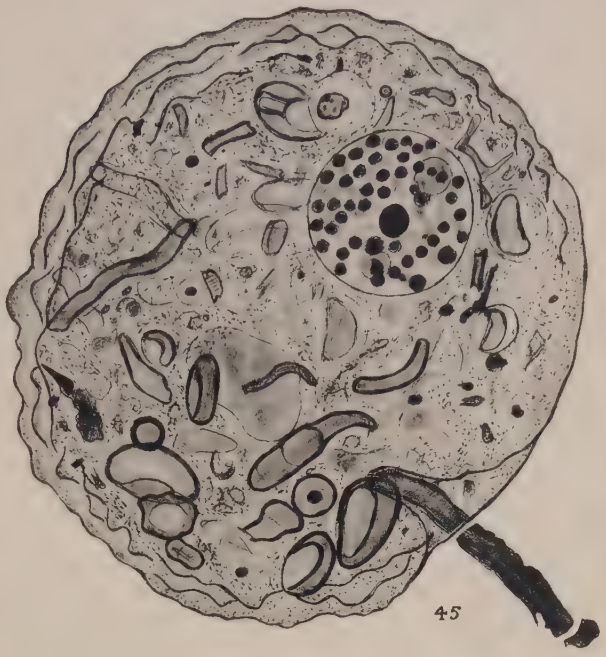

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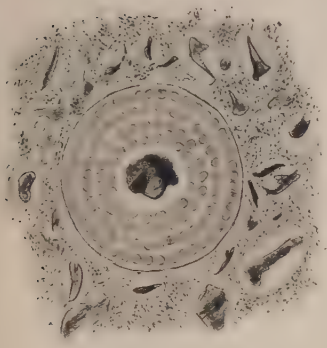

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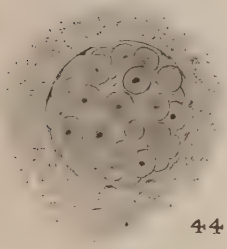

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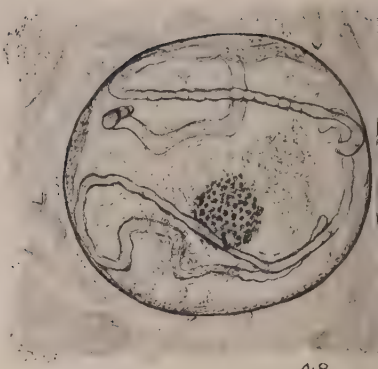

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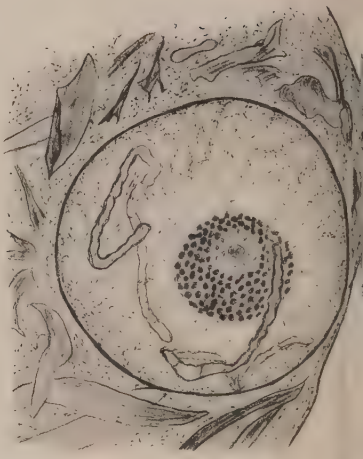

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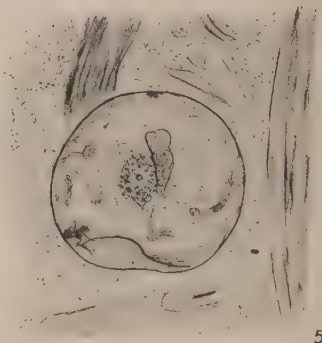

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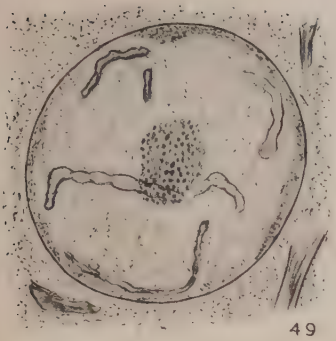

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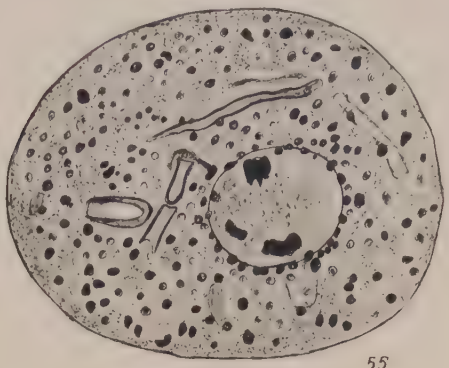

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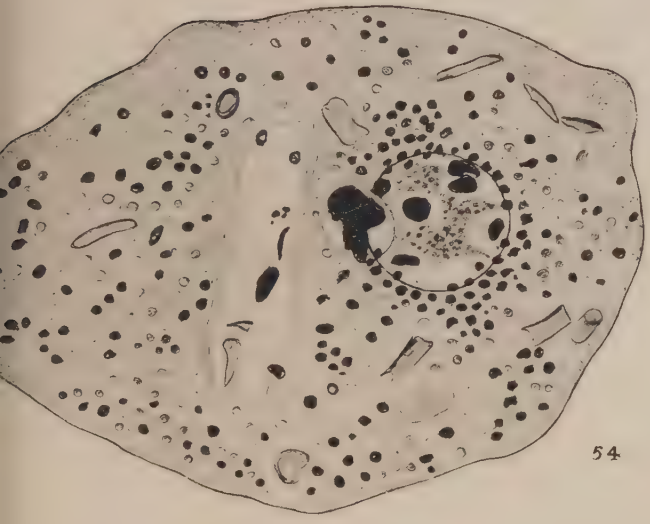

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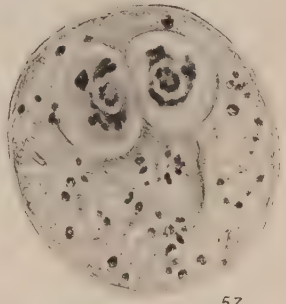

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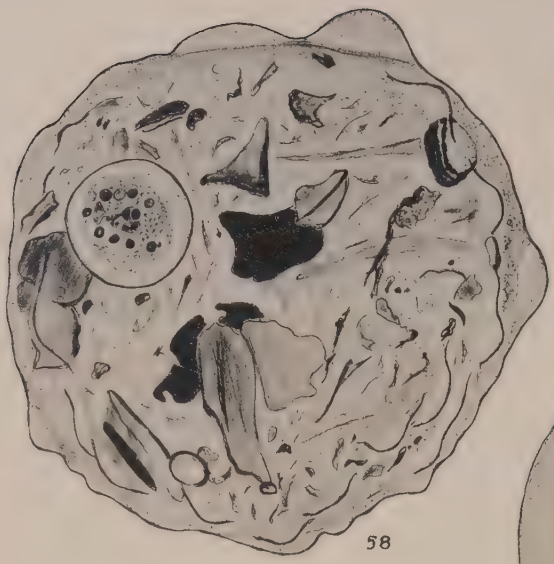

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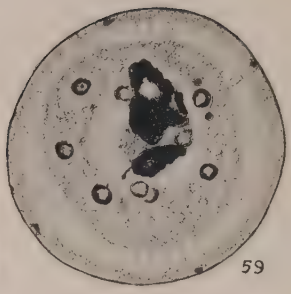

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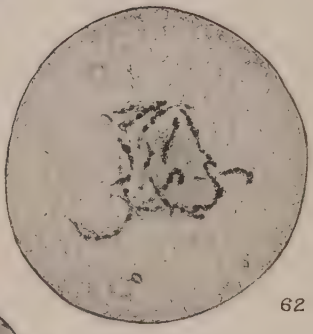

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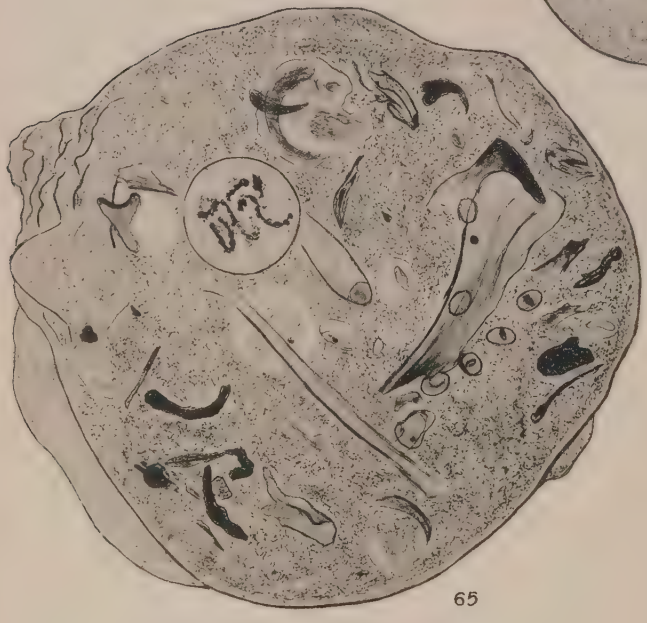

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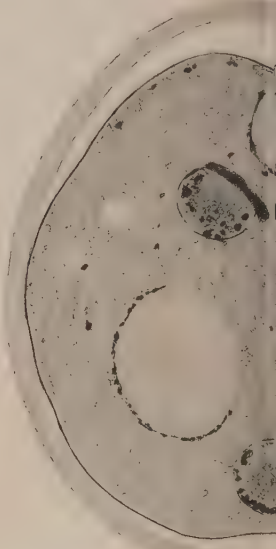

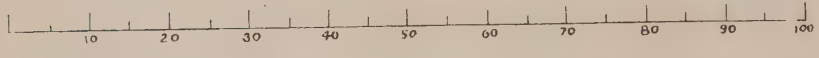

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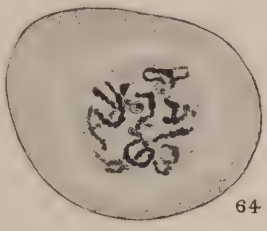

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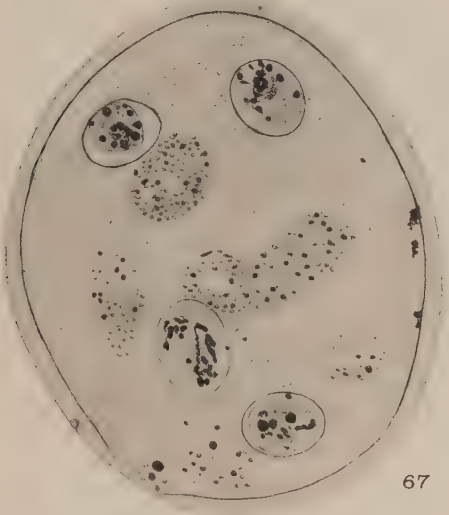

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THE NERVOUS SYSTEM OF PELAGIC NEMERTEANS.

WESLEY R. COE, Yale University.

During the past ten years a considerable number of species of bathypelagic nemerteans have been studied anatomically by Brinkmann ('17) and others and much information has been secured, showing the deviations of the nervous system from the general plan characteristic for the littoral forms. Five new genera, collected by the "Albatross" in various parts of the Pacific ocean, which have recently been fully studied by the writer (Coe, '26), proved of particular interest in this respect. One of these species, *Neuronemertes aurantiaca,* possesses a chain of ganglia along the dorsal nerve, a condition almost unique in invertebrates.

In the modifications of the sense organs also the pelagic nemerteans (Pelagica) differ widely from any of the littoral and bottom-living species (Reptantia).

It is to be recalled that bathypelagic nemerteans have been found only in the open oceans, where they swim with sluggish movements or float idly, but always far beneath the surface. Certain of the species are known to be restricted to particular water layers, usually at depths of from 500 to 1,500 meters, and presumably all the species are limited in their vertical range by the conditions of temperature and salinity, and to some extent, perhaps, by the pressure, to which they have become specifically adapted. Their geographical range, on the other hand, may be very wide, since water layers of essentially similar physical and chemical properties extend through vast areas of the oceans and almost from pole to pole. It is in conformity with these environmental conditions that the bathypelagic organisms have evolved.

Up to the present time 47 species of these aberrant worms have been described, but it is quite possible that some of them may be synonyms. More than half of them have been carefully studied with respect to their anatomical peculiarities, including those of the nervous system. Stiasny-Wijnhoff ('23) has also described

a form which was taken from the bottom at a depth of 833 meters, which is in some respects intermediate between the Pelagica and the Reptantia.

Brain.—In Fig. 1 a general plan of the nervous system is shown. The brain of the pelagic forms is essentially similar to that of the littoral species, but as a rule the dorsal ganglia are considerably smaller than the ventral. The two ganglia of each side are closely fused together. The fibrous cores and the three types of ganglion cells are also similar, but neurochord cells have not been found in any species.

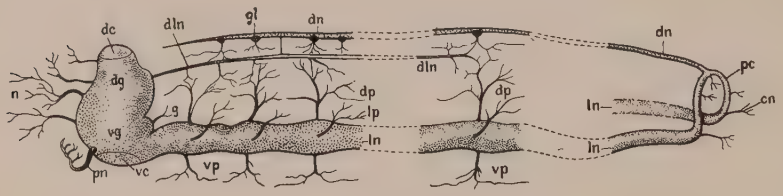

FIG. 1. Diagram of nervous system of *Neuronemertes aurantiaca* Coe, showing relation of brain and lateral nerves (*ln*) to peripheral nervous system; *dg*, dorsal, and *vg*, ventral ganglia of brain; *dc* and *vc*, the corresponding commissures; *n*, cephalic nerves; *g*, gastric nerve; *pn*, proboscidial nerve; *dn*, dorsal nerve, with metameric ganglia (*gl*); *dln*, dorsolateral nerve; *dp*, *lp*, *vp*, dorsal, lateral and ventral peripheral nerves, respectively; *pc*, posterior commissure; *cn*, caudal nerves.

One of the most striking peculiarities of the pelagic forms is the great amount of gelatinous tissue, or parenchyma, which surrounds all the internal organs, giving the body a high degree of permeability and a low specific gravity, enabling the worms to float freely in the water at great depths with a minimum of muscular exertion. This parenchyma surrounds the brain and usually separates it widely from the thin musculature of the head.

The probosis passes through the ring made by the ganglia and their dorsal and ventral commissures, while the stomach lies beneath the ventral commissure in most species.

Three longitudinal nerve stems extend the entire length of the body; the pair of lateral nerves, situated in the body parenchyma beneath the intestinal diverticula, and the median dorsal nerve, which lies directly beneath the basement layer of the epidermis. The former are direct continuations of the ventral brain lobes, while the dorsal nerve has no direct connection with the brain

(Fig. 1). The fibrous cores of these main nerves are connected at the posterior end of the body by the broad posterior commissure, which passes on the dorsal side of the rectum. They are also connected indirectly metamerically by the union of minute branches from both dorsal and dorsal peripheral nerves in the intermuscular plexus (Figs. 1, 2, 4).

Dorsolateral nerves.—In the anterior part of the body of many of the species studied a pair of nerves originating from the dorsal

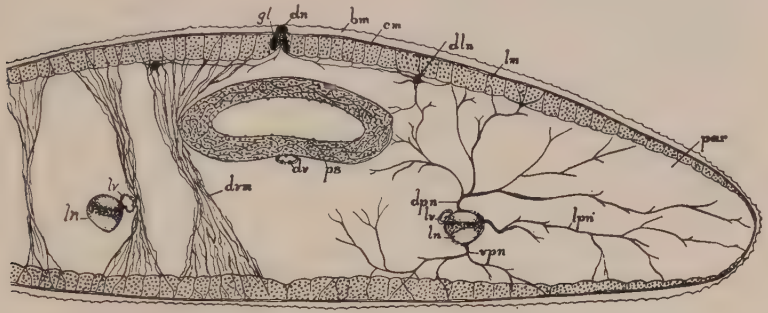

FIG. 2. Portion of transverse section of body of *Neuronemertes aurantiaca* Coe, showing on one side the relations of the peripheral nerves to the body musculature. The section passes between adjacent diverticula of the intestine and shows on the left hand side a portion of the dorsoventral musculature (*dvn*); *dn*, dorsal nerve, with bilobed ganglion (*gl*); *dpn*, *lpn*, and *vpn*, dorsal, lateral and ventral peripheral nerves; *ps*, proboscis sheath; *dv* and *lv*, dorsal and lateral blood vessels; *bm*, basement layer underlying the surface epithelium; *par*, parenchyma; *cm* and *lm*, the thin circular and longitudinal musculatures of the body walls. Other letters as in Fig. 1.

brain lobes, pass backward along the internal border of the body musculature near the dorsolateral edges of the probosis sheath. These dorsolateral nerves (Figs. 1, 2) are connected by means of delicate fibres with both the dorsal nerves and the dorsal periphereal nerves of the lateral nerve cords. Efferent branches supply the body musculature on the dorsolateral aspects of the body, as well as the proboscis sheath. These nerves are evidently homologous with the dorsolateral nerves of other Platyhelminths. They do not occur in all the pelagic species, and where present are limited to the anterior end of the body (Fig. 1). In other species, and toward the middle of the body in all species, their place is taken by fibres from the dorsal peripheral nerves.

Dorsal nerve.—This nerve appears to represent a local concentration of fibers from the dorsal peripheral nerves, for it is not connected directly with the brain, so far as known, in any of the pelagic forms. It extends in the median line and just external to the circular muscular layer, joining the commissure of the lateral nerves at the posterior end of the body. When followed forward toward the brain it becomes gradually smaller and finally terminates in the cephalic parenchyma or in the delicate intermuscular plexus a short distance posterior to the dorsal brain commissure (Fig. 1).

Fibers from the dorsal nerve supply the dorsal musculature and integument of the body. Other fibers enter the nervous plexus lying between the two muscular layers, while still others are connected with the dorsolateral and dorsal peripheral nerves.

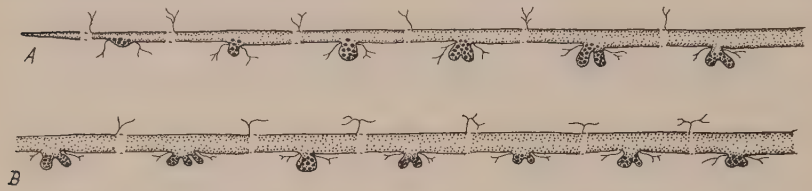

FIG. 3. *A,* Diagram of anterior end of dorsal nerve with its accompanying ganglia, showing variations in shape of the latter and the minute fibrils which pass dorsally into the integument and ventrally into the intermuscular plexus. *B,* Similar diagram from near middle of body.

Ganglia of dorsal nerves.—In most species only a few scattered ganglion cells are to be found along the course of the dorsal nerve, but in *Neuronemertes aurantiaca* this nerve is very large and is provided with a series of nervous structures, apparently of the nature of ganglia, although such organs have not hitherto been found in any species of nemertean. In my monograph on the Pelagic Nemerteans (Coe, '26) they were referred to as "problematical organs" although their probable nature as ganglia was emphasized (p. 130).

The number of these organs is upward of a hundred, corresponding presumably to a primitive metamerism.

Anteriorly the ganglia are somewhat smaller than the diameter of the fibrous core of the dorsal nerve, but farther back in the body they are several times as large as the nerve and are often

divided into two or more lobes (Figs. 3, 4). The nerve itself lies external to the circular muscular layer, while the ganglia are internal to these muscles and interposed between the bundles of longitudinal muscles. The broad connection between ganglion and nerve therefore requires the penetration of the thin circular musculature. This is accomplished by the mere separation of adjacent circular fibers so as to leave a narrow slit through which the connection passes.

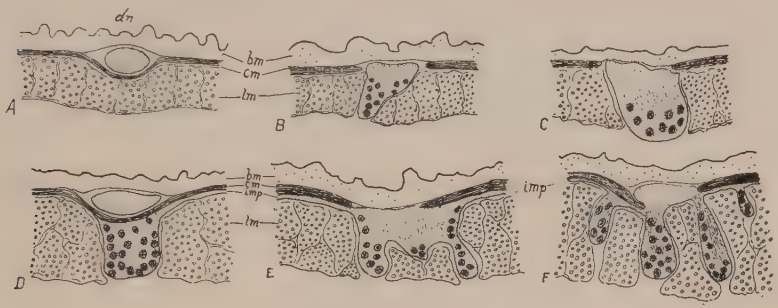

FIG. 4. Transverse sections of six of the ganglia of the dorsal nerve of *Neuronemertes aurantiaca* Coa, showing variations in shape and their relations to the nerve core. *A*, Dorsal nerve between two adjacent ganglia. *B*, Small ganglion closely fused with dorsal nerve. *C*, Large ganglion with ventral group of nerve cells. *D*, Surface view of ganglion, its connection with dorsal nerve being in an adjacent section. *E*, Large ganglion with three lobes of nerve cells and distinct intermuscular plexus. *F*, Large ganglion with four lobes of nerve cells; *imp*, intermuscular plexus; other lettering as in Fig. 2.

The cells composing the ganglia are similar in size and appearance to those which accompany the lateral nerve cords, and the cell boundaries are likewise rarely to be distinguished (Fig. 4). It has been difficult to determine the precise nature of these organs. That their component cells are the source of fibers which innervate the integument and musculatures seems highly probable. And it is also presumable that they supplement the nerve cells found in all species accompanying the lateral nerve cords, but there is no evidence that these latter cells are less abundant in this species than in other pelagic forms. A suggestion that may have some degree of plausibility is that they are associated with the great development of the dorsoventral musculature, particularly in the region where the spermaries are situated in the male,

just behind the head. In *Neuronemertes* the walls of the spermaries are not provided with the thick spiral musculature found in several other pelagic forms and which serves to supplement the weak muscles of the body walls in the forcible discharge of the spermatozoa. A similar result is accomplished however by the contraction of the highly developed dorsoventral muscles which closely invest the spermaries in that genus. It therefore seems reasonable to suspect that these supplementary nervous tissues may function in the control of this dorsoventral musculature throughout the body.

There still remains the possibility of the ganglia being correlated with sensory stimuli rather than with motor responses, but their structure is unlike that of any known receptor. It seems necessary, therefore, to return to the view that the ganglia of the dorsal nerve supplement the nerve cells of the lateral nerve cords just as these supplement those of the brain.

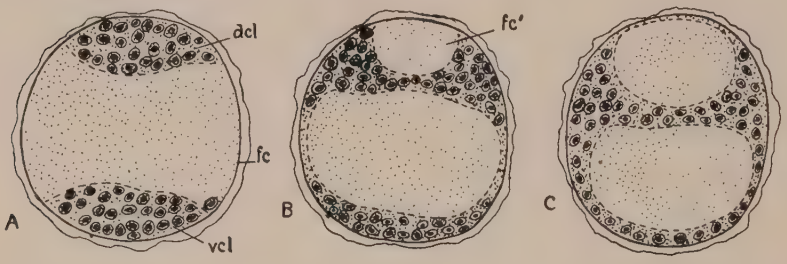

FIG. 5. Transverse section of lateral nerve cord in three different genera, showing the relations of the fibrous cores to the accompanying nerve cells. *A, Plionemertes plana* Coe, with single fibrous core (*fc*) and symmetrical dorsal and ventral cellular layers (*dcl* and *vcl*); *B, Proarmaueria pellucida* Coe, with small dorsal core (*fc'*); *C, Planonemertes lobata* Coe, with large dorsal core.

Lateral nerves.—These are direct continuations of the fibrous cores of the brain lobes and are accompanied by nerve cells throughout their entire length. At the posterior end of the body the cords of the two sides form a broad commissure on the dorsal side of the rectum (Fig. 1). In most pelagic species the fibrous core arising from the dorsal brain lobe is separated by a layer of ganglion cells, at least in the anterior part of the body, from that arising from the ventral lobe of the brain (Fig. 5).

The dorsal core is much smaller than the ventral and its separation from the latter tends to become less distinct in the posterior half of the body. Toward the posterior end of the body this core becomes gradually smaller, only the fibers of the ventral core being involved in the posterior nerve-commissure.

In a few genera there is but a single fibrous core, and this arises mainly from the ventral ganglion. It presumably also contains fibers from the dorsal ganglion, although they are usually not visibly separated from the others (Figs. 5, 8).

The position which the lateral nerves occupy in the body varies greatly according to the degree of development of lateral margins. In those forms in which the body is but little flattened, the nerve-cords lie near the lateral margins of the body, but in the broad, flattened forms they are far removed from the lateral margins, being in some cases situated more than half-way toward the median line (Fig. 6). In nearly all species the nerve-cords bend more or less sharply laterally immediately after their origin from the brain. In most forms they lie in the parenchyma near the ventral surface of the body, but in those cases where large ventral branches from the intestinal diverticulum force their way beneath the nerve-cord, the latter is moved dorsally until it may lie about midway between dorsal and ventral surfaces (Fig. 6).

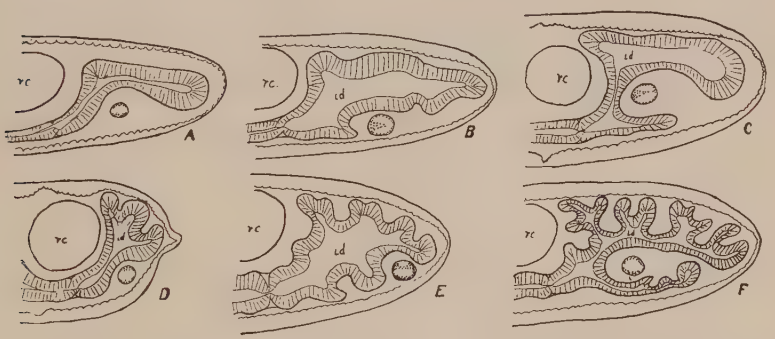

FIG. 6. Portions of transverse sections of body of six different genera, showing the position of the lateral nerve cord with respect to the body walls and intestinal diverticula. *A, Pelagonemertes joubini* Coe; *B, Planonemertes lobata* Coe; *C, Neuronemertes aurantiaca* Coe; *D, Cuneonemertes gracilis* Coe; *E, Nectonemertes pelagica* Cravens and Heath; *F, Planktonemertes agassizii* Woodworth.

Nerve cord muscle.—In the genera belonging to the families *Armaueriidæ* and *Pelagonemertidæ* and in *Pendonemertes* of the *Protopelagonemertidæ* a narrow band of muscular fibers splits off from the proboscis sheath on each side of the body immediately back of the brain and extends backward throughout the length of the body in close connection with the lateral nerve cord. In some forms it lies close against the outer connective sheath on the median face of the nerve cord, while in other cases it penetrates the sheath and comes to lie in close connection with the nervous tissues. This muscle is usually very thin, consisting of only one or two layers of muscular fibers (Figs. 7, 8*A*), but in a few species it is so large that it equals fully half the diameter of the nerve cord itself (Fig. 8*B*).

In *Pelagonemertes joubini* the muscle leaves the proboscis sheath as several separate strands (Fig. 7) and these may at first take separate positions on the face of the nerve cord, but further back in the body they fuse into a single flattened bundle.

The function of the nerve cord muscle is evidently to hold the nerve cord in position with reference to the organs which the latter innervates.

Peripheral nerves.—As a rule three peripheral nerves leave each lateral nerve cord in each of the spaces between adjacent intestinal diverticula. One of these three nerves, dorsal peripheral, leaves the dorsal side of the cord (Figs. 1, 2, 7, 8) to supply the dorsal musculature and integument and in some cases the proboscis sheath (Figs. 2, 7). This nerve also sends branches into the intermuscular plexus and indirectly connects with the dorsal and dorsolateral nerves. Small branches also supply many of the dorsoventral muscles.

The lateral peripheral is a slender nerve with few branches. These supply the lateral margin of the body and appear also to enter the intermuscular plexus (Figs. 2, 7).

The ventral peripheral originates from the ventral core of the cord and divides into two branches which supply the ventral portions of the body. Branches from this nerve also enter the ventral intermuscular plexus (Figs. 1, 2, 7, 8).

Intermuscular plexus.—In some of the sections of *Neuronemertes aurantiaca* distinct nerves can be seen leading from the

dorsal nerve inward through the circular muscular layer and thence laterally between the two muscular layers of the body-walls. These nerves appear to constitute part of a great plexus

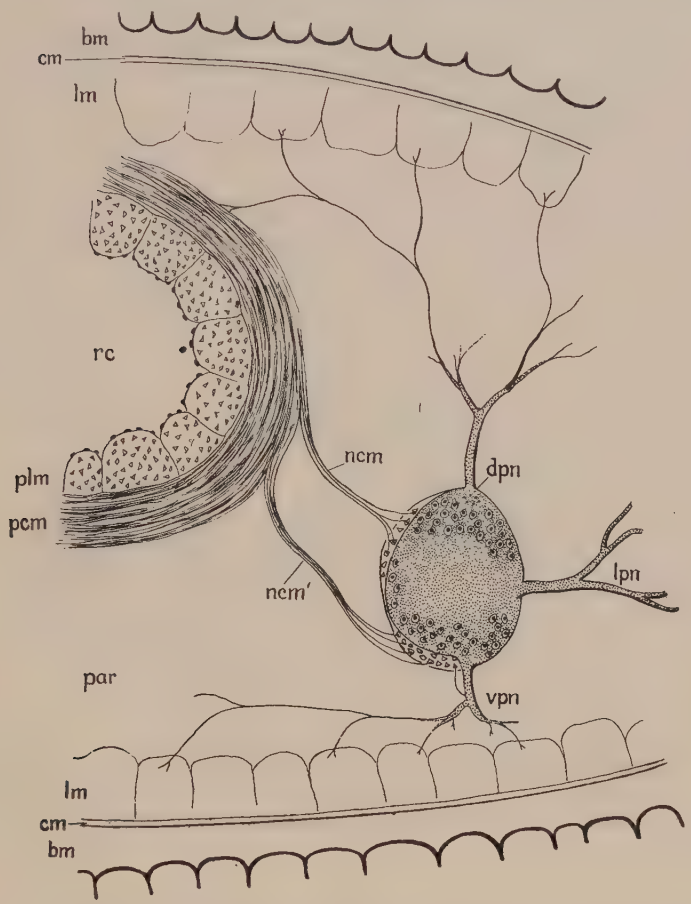

FIG. 7. Diagram of portion of transverse section of body of *Pelagonemertes joubini* Coe, immediately posterior to the brain, showing origin of the nerve cord muscles (*ncm, ncm'*) from the outer spiral musculature of the proboscis sheath, and the three main branches, dorsal (*dpn*), lateral (*lpn*), and ventral (*vpn*) which leave the nerve cord in each interdiverticular space; *rc,* rhynchocoel; *plm* and *pcm,* longitudinal and circular-spiral muscular layers of proboscis sheath; other lettering as in Fig. 2.

of nerve-fibers which extends between the circular and longitudinal musculatures throughout the length of the body, both

dorsally and ventrally (Fig. 4). The plexus is in frequent communication with the peripheral nerves from the lateral cords, as well as with the dorsal and dorsolateral nerves.

Tentacular nerves.—The adult males of *Nectonemertes* and both sexes of *Balænanemertes* are each provided with a pair of tentacles immediately back of the head. These appendages are lateral outgrowth of the body walls and are supplied with large nerves homologous with the lateral peripheral nerves in other parts of the body (Fig. 9).

Proboscis-sheath nerves.—In many pelagic species the proboscis sheath is supplied with nerves which enter it at the ring where the proboscis is attached, but in other forms the organ also receives one or more pairs of fine branches from the dorsal peripheral nerves (Figs. 2, 7).

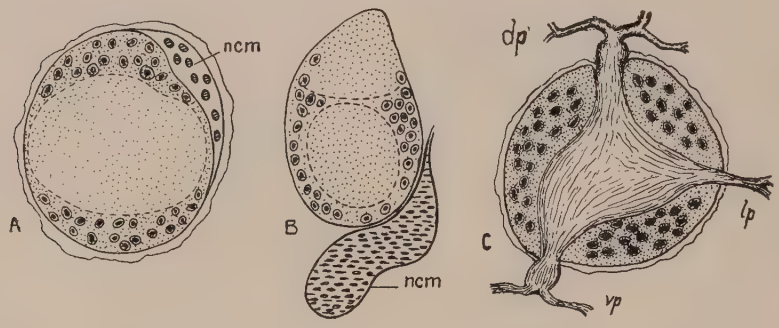

FIG. 8. *A,* Transverse section of lateral nerve cord and accompanying muscle (*ncm*) in *Cuneonemertes gracilis* Coe. *B,* Same in *Balænanemertes chuni* Bürger. *C,* Section of lateral nerve cord in *Planktonemertes agassizii* Woodworth, showing the origin of dorsal lateral and ventral peripheral nerves.

Proboscidial nerves.—The proboscis is large and highly specialized in most of the pelagic nemerteans, with a powerful musculature and a thick epithelial lining of secretary and sensory cells in addition to the minute sickel-shaped weapon with which it is provided. Consequently it requires an abundant supply of nerves. In some forms these are provided by means of a pair of large nerve stems arising from the points where the ventral commissure joins the ventral brain lobes (Fig. 1). This pair of large nerves divides into the definite number of proboscidial nerves

either at the attachment of the proboscis or in the organ immediately posterior to its insertion. But in other forms, where the proboscis is inserted close in front of the brain, the proboscidial nerves either arise in the complete number from the ventral brain lobes and the commissure adjacent or branch immediately after their origin.

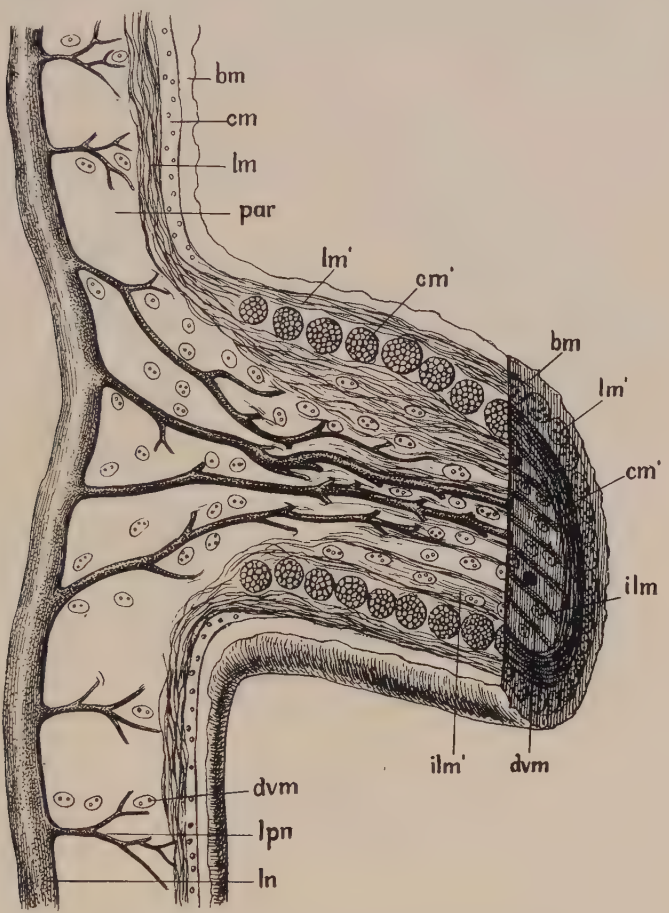

FIG. 9. Diagram of portion of horizontal section of tentacle and adjacent body wall of *Nectonemertes mirabilis* Verrill, showing the four large tentacular nerves originating from the lateral nerve cord (*ln*). These are homologous with the lateral peripheral nerves (*lpn*) of the rest of the body; *bm*, basement layer underlying the surface epithelium; *lm*, longitudinal and *cm*, circular muscular layer; *ilm*, internal longitudinal muscles; *dvm*, dorsoventral muscles; *par*, *parenchyma*.

The nerves take up a position in the midst of the longitudinal muscular layer along the whole circumference of the proboscis (Fig. 10). This position varies somewhat in the different forms, but is generally about two thirds the distance from the inner to the outer border of the longitudinal muscular layer. Here the nerves branch out to form a thick plexus of connecting fibers and send symmetrical branches radially both towards the periphery and towards the glandular epithelium lining the central lumen (Fig. 10).

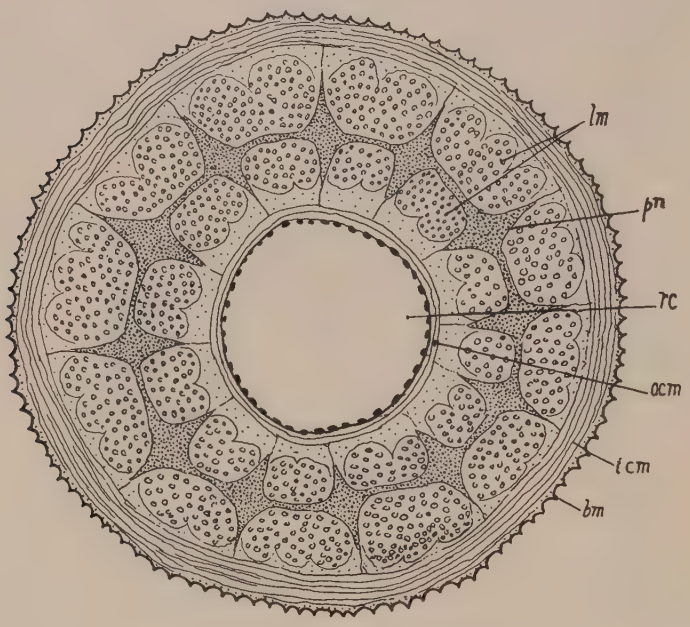

FIG. 10. Transverse section of everted proboscis of *Cuneonemertes gracilis* Coe, with the 12 proboscidial nerves (*pn*) connected by a plexus in the midst of the longitudinal muscular layer (*lm*); *icm* and *ocm*, inner and outer circular musculatures; *bm*, basement layer underlying the inner, grandular epithelium; *rc*, extension of the rhynchocoel; lined with endothelium.

About midway between each two proboscidial nerves in certain species the plexus is condensed into a secondary nerve, commonly about half the diameter of one of the main nerves. There is thus an equal number of primary and secondary nerves in such species. In some forms it is difficult to distinguish the two sets

of nerves, so that one specimen may appear to have double the number found in other specimens where the proboscis is in a different state of contraction. Furthermore in some forms the number of nerves in the anterior end of the proboscis may be double that found farther back toward the stylet region, due to the gradual disappearance of each alternate, or secondary, nerve in the plexus. And, finally, the number may vary to some extent in different individuals, as in *Nectonemertes mirabilis* where the number ranges from eighteen to twenty-four.

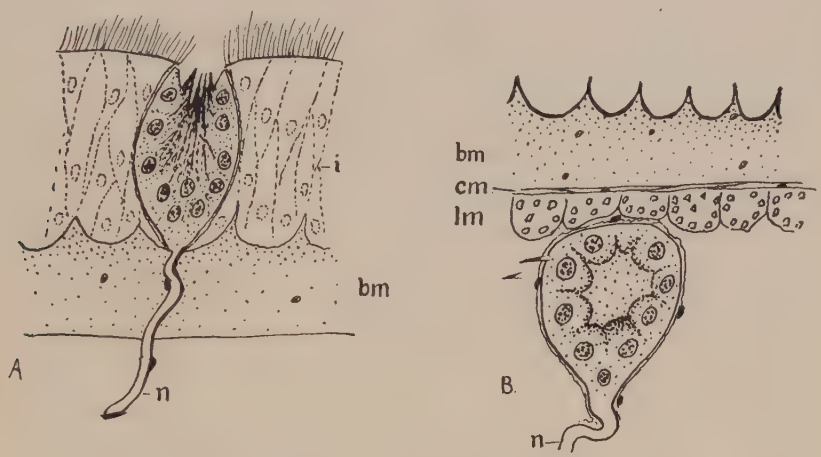

FIG. 11. Diagrams of the two types of sense organs found in *Cuneonemertes gracilis* Coe. *A,* integumentary sense organ; *B,* subcutaneous sense organ; *bm,* basement layer of surface epithelium (*i*); *cm* and *lm,* circular and longitudinal musculatures of cephalic wall; *n,* nerve leading to dorsal brain lobe.

The central radial branches from the nervous plexus pass between the bundles of longitudinal muscles and form a second, delicate intermuscular plexus beneath the inner circular muscles. From this plexus fine nerve-fibers supply the epithelium of the papillæ as well as the inner circular muscles.

The peripheral radial branches arising from each of the primary nerves form a third, still more delicate, plexus between the longitudinal muscles and the outer circular muscular layer. This supplies in a similar manner the more superficial layers of the proboscis, namely, the outer circular muscles and the endothelium.

Of the three nervous plexuses (middle, inner, and outer) which are thus formed the two latter are of such great delicacy that they can be observed only under favorable conditions of preservation, and it is by no means certain that they are present in all species.

Although the radial nerves leave the middle plexus only at intervals, the three plexuses appear to form more or less continuous networks throughout the length of the anterior chamber. In some forms even the middle plexus is incompletely developed, the primary proboscidial nerves being connected with each other only at intervals.

The plexuses are interrupted in the middle chamber of the proboscis by the interlacing of the muscular layers, and the primary nerves gradually lose their identity in a multitude of smaller branches, some of which innervate the stylet musculature and gland-cells, while others continue backward into the wall of the posterior chamber. Hence in the posterior chamber the primary nerves are not usually distinguishable as separate units and there is but a single plexus lying between the longitudinal muscles and the glandular epithelium lining the lumen.

The smallest number of proboscidial nerves found in any pelagic species is 7, and the largest is 50, the most frequent numbers being from 15 to 25. In general, the species in which the individuals attain the largest size have a greater number than those in which the animals are but a few millimeters in length. Odd and even numbers are about evenly distributed.

Cephalic nerves.—The muscles, integument and sense organs of the head are supplied with a considerable number of fine nerves which arise from the anterior surfaces of both dorsal and ventral brain lobes (Fig. 1). But since the cephalic musculature is extremely weak and the sense organs rudimentary, the cephalic nerves are very much smaller than in most other nemerteans.

Gastric nerves.—A pair of small nerves arise from the posterior end of the ventral brain lobes to supply the anterior end of the digestive tract (Fig. 1). These are homologous with the esophageal nerves of the littoral forms which have the esophagus well developed, but where that part of the digestive system is rudimentary, as in the pelagic species, the nerves supply the stomach. Supplementary gastric nerves, originating from the ventral commissure, have also been observed in one or more species.

Pyloric nerves.—In some species, at least, a pair of minute nerves, originating from the lateral nerve cords, enters the anterior end of the pylorus.

Caudal nerves.—The posterior extremity of the body is supplied with nerves in two different ways. In *Proarmaueria* the posterior end of each of the lateral nerves divides into two branches, the larger of which enters the posterior commissure, while the smaller supplies the extremity of the body. In *Neuronemertes* and some other forms the caudal extremity is supplied with small nerves from the commissure itself (Fig. 1).

Sense-organs.—Even the most primitive of the littoral nemerteans are provided with special sense-organs as differentiated portions of the integument, and in the higher groups ocelli, cerebral sense-organs, and frontal sense-organs are of frequent occurrence. In addition, otocysts are found in a few species. But in the pelagic forms no such organs have been found in any of the species studied. Possibly vestiges of one of these types of sense-organs were discovered by Bürger ('09) in *Balænanemertes.* These are considered by him to be rudimentary ocelli, and similar organs were shortly afterward found by Brinkmann in *Pelagonemertes rollestoni.* Further studies have shown them to be widely distributed among the *Armaueriidæ* and *Pelagonemertidæ.* They are of two distinct types, as follows:

Subcutaneous sense-organs.—In these forms a small cluster of sense-organs, whatever their nature may be, is situated beneath the basement layer of the anterior margin of the head, frequently on either side of the rhynchodeal opening. Each of the end-organs consists of a pear-shaped group of cells, connected with a branch of one of the cephalic nerves (Fig. 11*B*). Some of them lie in contact with the inner border of the basement-layer, while others may be situated somewhat deeper in the cephalic tissues, and in or even beneath the muscular layers. Since they differ widely from typical nemertean ocelli in structure, it seems quite possible that these may be special sense-organs peculiar to the pelagic forms rather than that they represent degenerate ocelli.

Integumentary sense-organs.—A second type of sense-organ occurs in the integument of the head and less frequently in other parts of the body in certain genera. These consist of compact

groups of sensory cells imbedded in the integument. The cells are provided with slender distal processes and each group is supplied with a nerve leading to the brain or to the lateral nerve, according to its location (Fig. 11*A*). It is not improbable that such integumentary sense-organs occur in the majority of the pelagic species, but that the loss of the integument during capture has prevented their discovery.

Summary.

Correlated with their usually gelatinous consistency, weak musculature and other adaptations for life far beneath the surface of the oceans, the nervous system of the bathypelagic nemerteans (Pelagica) differs considerably from that found in the littoral and bottom-living relatives (Reptantia). Comparisons are made between the Pelagica and Reptantia with respect to each of the principal parts of the nervous system. The special sense-organs of the Reptantia are either lacking entirely or merely vestigial in the bathypelagic forms. With particular reference to a recently described species, *Neuronemertes aurantiaca:*

1. The dorsal nerve is not connected directly with the brain.

2. The dorsal nerve is provided with metameric ganglia, not previously known for any nemertean.

3. A pair of dorsolateral nerves connects both with the dorsal nerve and with dorsal peripheral branches of the lateral nerve cords.

4. A delicate intermuscular plexus lies between the two body musculatures.

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DISCUSSION AND COMMUNICATIONS.

THE SCRIPPS INSTITUTION OF OCEANOGRAPHY OF THE UNIVERSITY OF CALIFORNIA.

On the shore of one of the most picturesque of the little bays on the coast of Southern California and two miles from the beautiful city of La Jolla is situated the only oceanographic institution in America and one of the few of its kind in the world. This locality is particularly favorable for research on the sea and its inhabitants, not only because of its natural physical charms, with towering cliffs undermined by the action of the waves and eroded into caverns, with adjacent alluvial plain and rugged mountains, level mesas and precipitous canyons, but also because it has easy access to the greatest variety of marine topography. Muddy bays and sandy beaches, rocky tide pools and immense kelp beds, with an ocean floor descending rapidly to considerable depths, are near at hand. Its climate, too, is unsurpassed and conducive to sustained activity both in summer and in winter.

The institution is the outgrowth of the Marine Biological Station of the San Diego Natural History Society. Supported by generous gifts from members of the Scripps family, and including 177 acres of land, the station became a part of the University of California in 1912, under the directorship of William E. Ritter. It was then known as the Scripps Institution for Biological Research and in its earlier years the activities of the staff of investigators were devoted primarily to marine biology and animal genetics, with some attention to the physics and chemistry of the ocean water.

During these years a preliminary biological survey was made of the coastal and off-shore waters, and a large number of previously undescribed species of the various groups of marine organisms were made known to science. Particular attention was given to the plankton. During this period also physico-chemical and meteorological data were collected and the foundations laid for the more extended work in these fields which has been prosecuted uninterruptedly until the present time.

With the retirement of Director Ritter and the appointment of the present director, Dr. T. Wayland Vaughan, the scope of the investigations became more preponderantly of an oceanographic character and in 1925 the name of the institution was changed to its present title, which is indicative of the future program of the institution's activities, embracing all aspects of oceanographic research, some of them being of very great economic importance.

Ample facilities for many of these investigations are already available. A two-story laboratory building with the necessary

aquaria and an abundance of pure sea water is well equipped for a dozen or more investigators; a library-museum building with an exceptionally complete series of journals, particularly in zoology; a concrete pier, 1,000 feet in length; a 65-foot boat of 22 tons capacity, which gives ready access, in this region of placid seas, to ocean depths of considerable magnitude; modern apparatus for obtaining temperature records, bottom samples and plankton to a depth of 1,000 fathoms; a hydrographic and meteorological station at the end of the 1,000-foot pier, with thermographs for surface and bottom temperatures. A second thermograph is carried on a liner plying between San Francisco and Honolulu, while a third is established at Balboa, California. With these instruments an extended test as to the correlation of ocean temperatures and subsequent precipitation on the California coast is to be made. A seismograph is installed for recording the oscillations of the earth's crust in this region of surface instability.

Hydrographic data and water samples for analysis are daily taken at several points on the coast, between San Diego and Alaska, while naval transports and lighthouses supply extensive hydrographic and meteorological data. The tabulation of these observations through a series of years, correlated with similar records collected by Japan and other countries bordering the western Pacific, promise to shed much light on the origin and movements of cyclonic disturbances approaching our western coast and determining the weather conditions over the entire country.

Some of the other economic problems within the sphere of investigations of the institution relate to the fisheries and other industries dependent upon marine products, the origin of useful substances through geological agencies on the ocean floor, the cause and course of ocean currents as affecting commerce and navigation, and a multitude of allied topics. The chemistry and physics of the sea form the basis for all investigations of this nature, and these fundamental problems are receiving special consideration.

Much attention has been given to the plankton, not only because of its scientific interest but also because of its economic importance in forming the elemental materials upon which the food supply of fishes directly or indirectly depends, while a major investigation dealing with animal genetics has been in progress from the early days of the institution and has yielded results of wide biological significance.

The published contributions to science by the distinguished group of workers who have been connected with the institution form a record of remarkable activity and progress. The results of the research by members of the regular staff embrace more than 200 papers and monographs, while numerous investigators

from all sections of the United States and from several foreign countries, who have found here the opportunities for solving some of their most important problems, have published many more. The institution itself maintains its own series of bulletins, although a large portion of the work of the staff is published in other journals.

Finally, though by no means the least important, are the provisions for the physical welfare of the investigators. Groups of comfortable cottages overlooking the blue Pacific are provided for the members of the staff and for visiting scientists at a nominal rental—an intellectual colony with an atmosphere highly conducive to successful accomplishment. The writer after nearly a year in these inspiring surroundings takes pleasure in recording the cordial hospitality afforded to the visiting investigator and the generosity with which the abundant facilities of the institution are placed at his disposal.

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THE COMPARATIVE EFFECT OF ETHYL CYANIDE AND ETHYL ISO-CYANIDE ON BIOLOGICAL OXIDATIONS

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While much work has been done on the effect of hydrocyanic acid and its salts on cellular oxidations, relatively little has been done with the organic cyanides and still less with organic iso-cyanides. The physiological action of these is usually dismissed by saying that they have a singularly persistent and disagreeable odor and are highly toxic. Accordingly we have undertaken to compare the effects of ethyl cyanide and ethyl iso-cyanide on the oxygen consumption of living organisms of a simple type.

The structure of potassium and other inorganic salts of hydrocyanic acid is still a matter of some doubt. Some evidence indicates that the structure of potassium cyanide is K C:N; the bulk of evidence, however, indicates that the structure is K N:C. Consequently, conclusions drawn from the effects of potassium cyanide on oxidations are limited in value by the fact that the exact structure of the salt is not definitely known. On the other hand, ethyl cyanide is known with certainty to have the formula C2H5C:N, while Nef has shown that ethyl iso-cyanide has the structure C2H5N:C. The carbon atom in the iso-cyanide group is bivalent; it is therefore highly unsaturated and extremely reactive. The cyanide group is also very reactive, due to the triple bond between carbon and nitrogen, but the saturation and activity of this group is somewhat less than in the iso-cyanides.

We prepared ethyl iso-cyanide by the action of silver cyanide on ethyl iodide, according to the directions of Gautier (1). Using 115 grams of ethyl iodide and 100 grams of silver cyanide, Gautier

obtained 19 grams of ethyl iso-cyanide. Our yield was somewhat less. We saved the product boiling between 77° and 78.5°. It is a colorless, mobile liquid but turns brown after standing a few weeks, even though kept tightly stoppered. It has a frightful odor even in extreme dilutions.[1]

We prepared ethyl cyanide by dehydrating propionamide with phosphorus pentoxide. The product so obtained boiled between 96° to 98° and had a pleasant aromatic odor without a trace of its fearful smelling isomer. Our solutions of these substances were prepared by dissolving a weighed amount of the substance in a liter of tap water and diluting with tap water to the appropriate concentration. Both the cyanide and the iso-cyanide are very volatile, but since we made up at least 4 liters of each solution at a time and used them at once, the error introduced by the evaporation of the substances was undoubtedly small and as it occurred in both the normal and iso-cyanide solutions, may be disregarded. A considerable waste of our cyanides was unavoidable, however.

Since we could not use strong concentrations because of our limited supply of ethyl iso-cyanide and the very considerable difficulty in handling strong solutions of this substance without specially devised apparatus, it was essential to employ as the living material on which to test the comparative effects on oxygen consumption of the two types of cyanide, organisms which are highly susceptible to cyanide action. Previous work by Allen (2), Hyman (3) and one of us (J. W. B. (4)) has shown freshwater flatworms to be very sensitive to extremely dilute solutions of KNC. From this fact we were lead to expect that they might show considerable sensitivity to depression by organic cyanides. Additional advantages that influenced us to employ freshwater flatworms were that we would thus eliminate complications which would be expected in more complex organisms, due to the effects of the agents on highly organized nervous systems and blood streams. Furthermore, satisfactory methods of handling these

[1] Through the kindness of Capt. T. J. Johnson, C.W.S., U.S.A., we were given the use of two type 1-2-2 Army gas masks, which were very effective and indispensable in handling this fearfully smelling substance.

animals in oxygen consumption tests have been worked out and we were therefore spared extensive preliminary experiments which would otherwise have consumed much of our valuable iso-cyanide Hence we used two species of freshwater flatworms, Planaria dorotocephala and Planaria maculata. Since the effects of KNC on Planaria are known (2), (3), (4), we are able to make a general comparison between its effects and those of the organic cyanides which we employed.

Our general method consisted in measuring the oxygen consumption of two comparable lots of animals in water over a convenient period, then replacing the water over one of the lots with the desired concentration of normal ethyl cyanide and over the other lot with an iso-molal concentration of ethyl iso-cyanide and determining the oxygen consumption of the animals in these solutions. The animals were then weighed and the results calculated in terms of cubic centimeters of oxygen consumed per milligram of tissue per two hours. All solutions were made up in tap water and adequate oxygen content was assured by shaking the solutions thoroughly with air. Temperature was controlled by immersing the flasks containing the animals in a water bath and maintaining the temperature of the bath constant within one-half degree throughout the experiment. As in the previous work of one of us (J. W. B.), the oxygen content of the water and of the solutions was determined by the Winkler method. The exact procedure is described in detail elsewhere (5) and will not be re-described here. The limitations of our supply of the iso-cyanide restricted us to a relatively small number of experiments. However, the results are consistent and free from exceptions; when treated biometrically they show distinct differences in the effects of the agents.

As preliminary experiments we tried the effect of M/10,000 solutions of both substances on the oxygen consumption of Phagocata gracilis, a genus closely related to Planaria. Two experiments failed to show any effects. Similarly, two experiments with M/25,000 solutions on Planaria dorotocephala yielded no evidence of depression of oxygen consumption by either agent. Using M/10,000 solutions on Planaria dorotocephala, however, we

obtained evidence of depressive effects. Similar results were obtained with this concentration on Planaria maculata. Hence we used this concentration and these two forms. In two experiments M/5000 solutions were applied. M/5000 solutions of ethyl iso-cyanide are extremely unpleasant to handle, so we did not extend this series of experiments.

TABLE 1

NUMBER	AGENT	CONCENTRATION	TEMPERATURE	DURATION OF EXPOSURE	CONSUMPTION IN WATER	CONSUMPTION IN AGENT	EFFECT
			°C.	hours			per cent
1	Norm.	M/10,000	22	10	0.00049	0.00047	−4
2	Iso.	M/10,000	22	10	0.00046	0.00040	−13
3	Norm.	M/10,000	22	8	0.00060	0.00056	−6
4	Iso.	M/10,000	22	8	0.00058	0.00052	−10
5	Norm.	M/10,000	22	8	0.00085	0.00082	−3
6	Iso.	M/10,000	22	8	0.00080	0.00071	−11
7	Norm.	M/10,000	19	20	0.00054	0.00054	0
8	Iso.	M/10,000	19	20	0.00048	0.00044	−8
9	Norm.	M/10,000	19	20	0.00054	0.00056	+3
10	Iso.	M/10,000	19	20	0.00043	0.00039	−9
11	Norm.	M/5,000	20	8	0.00057	0.00056	−1
12	Iso.	M/5,000	20	8	0.00048	0.00043	−10
13	Norm.	M/5,000	20	8	0.00058	0.00061	+5
14	Iso.	M/5,000	20	8	0.00041	0.00035	−14

Mean effect of Normal	−0.85 ± 0.929 per cent
Mean effect of Iso	−10.7 ± 0.507 per cent
Mean of difference	9.85
Standard Deviation of difference	4.32
M/S.D.	2.28
Probability	0.99928
Odds	1388 to 1

Oxygen consumption in terms of cubic centimeter per milligram per two hours. Experiments 1, 2, 3 and 4, Planaria dorotocephala; all others, Planaria maculata.

Forty or more animals were employed in each test. The containers were Erlenmeyer flasks, capacity 500 cc. The duration of exposure to the cyanides varied in different experiments from eight to twenty hours. Since the total weight of the forty animals in each flask was in no case greater than 400 mgm., and the capacity of the flasks 500 cc., effects due to accumulation of

waste products in important concentration, to lowered oxygen supply, and to withdrawal of the cyanides by the animal tissue, are to be regarded as negligible.

Our results are given in full in table 1. They show that as regards the normal ethyl cyanide there is no appreciable effect of the concentration used on oxygen consumption, for the probable error of the mean is greater than the mean itself. In experiments 1, 3 and 5, the normal ethyl cyanide used was obtained from a chemical supply house and was of the grade known as c.p. Its boiling point varied from 88° to 120°. In experments 7, 8, 11 and 13, the normal cyanide was made by one of us (O. H. E.); after re-distillation its boiling point was 96° to 98°. The results indicate that the commercial product tends to produce depression in the concentrations used, due, perhaps, to its containing impurities in the form of methyl and propyl cyanide.

As regards the effect of ethyl iso-cyanide, the results show clearly that in the concentrations employed it has a depressive effect on oxygen consumption, the mean effect being more than twenty times the probable error. That there is a significant difference between the effects of the normal and the iso-cyanide is further strongly indicated by the calculation of the mean difference and its standard deviation. According the table of "Student" (6) when M/S.D. is 2.28, and N is 7, then the probability is 0.99928, or the odds are 1388 to 1 that the iso-cyanide is the more effective depressant.

So far as our results go, they indicate that the action of ethyl iso-cyanide is similar to that of KNC, but much less pronounced. As is the case with KNC (3), the depressive effects of the iso-cyanide is not correspondingly increased by doubling the concentration. Furthermore, its effect resembles that of KNC in that the degree of depression is independent of the duration of exposure. But ethyl iso-cyanide is not so powerful depressant of biological oxidations as KNC. M/25,000 solutions of KNC produce a mean depression of oxidations in Planaria dorotocephala of 57.8 ± 0.527 per cent (4). According to the results recorded here, the depressive effect of M/10,000 and M/5000 ethyl iso-cyanide on oxidative metabolism in the same and a closely related species of Planaria is but 10.7 ± 0.507 per cent.

The relative toxicity and relative depressive action of various members of the nitril series have received attention by a number of investigators, notably Verbrugge (7), Mathews and Walker (8), Warburg (9), and Skanischewsky (10). According to Mathews and Walker, the less stable members are the more toxic, or the more powerful depressants. So far as we are aware, comparisons between iso-molal solutions of the nitrils and iso-nitrils have not heretofore been made.

SUMMARY

In iso-molal solutions, ethyl iso-cyanide is a more powerful depressant of oxygen consumption in Planaria than is normal ethyl cyanide. The effects of ethyl iso-cyanide resemble those of potassium cyanide.

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SOME LIMITATIONS OF WARBURG'S THEORY OF THE ROLE OF IRON IN RESPIRATION

The construction of models of biological processes has occasionally contributed greatly to the knowledge of the mechanisms of vital phenomena. However, enthusiasm over the successful construction of a model that in part duplicates the reaction of living protoplasm often obscures the fact that the duplication is only partial and misleads the investigator into undue dependence on deductive reasoning. Caution must be observed in accepting theories of the organism derived from the behavior of models, for in attempting to isolate a single process in this manner the controls and correlations that distinguish the living from the non-living are lost. Obviously, the value of any theory derived from the behavior of a model depends on the extent of resemblance between the behavior of the non-living system and the facts and characteristics of the process of the living organism which it purports to simulate.

An examination of the characteristics of biological oxidations shows that the comprehensive theory of Warburg of the mechanism of oxidations in the cell,[1] based largely upon the characteristics of his so-called models of respiration, is not free from the criticisms that have been levelled at other theories of biological

[1] Warburg, O., 1921, *Biochem. Zeitschr.*, cxix, 134; 1923, *ibid.*, cxxxvi, 266; 1923, *ibid.*, cxlii, 518; Science, n. s., 1925, lxi, 575.

processes similarly derived from models. On the contrary, the divergence between Warburg's theory and the actual facts is wide enough to justify regarding the theory as distinctly limited in application.

Briefly, Warburg has developed a theory of cellular oxidations in which iron in unknown combination with nitrogen is held to play the rôle of catalyzer. Molecular oxygen is said to enter into combination with the iron to form higher oxides of iron, and the iron-nitrogen is assigned the property of adsorbing and peculiarly loosening the bonds of amino acids in the cell. In the oxidation of carbohydrates phosphates replace nitrogen, and in the oxidation of fats the presence of the SH group is necessary. A transfer of active oxygen is thus effected, and the iron is returned to a lower oxide. Unfortunately for the theory in its present form, the facts of cellular oxidations which Warburg cites in support of his theory are in some important details disputable.

The theory demands, and Warburg has shown in the case of the unfertilized sea urchin egg,[2] that the oxygen absorption of disintegrated cells is equal to that of intact cells. This is contradictory to sound evidence[3] which shows that the chief energy-releasing oxidations in the cell are profoundly depressed by mechanical destruction of the cellular structure. This contradiction between the findings of Warburg and those of others shows that the experimental result

[2] Warburg, O., 1911, *Zeitschr. f. physiol. Chem.*, lxx, 413; 1914, *Arch. f. ges. Physiol.*, clviii, 189.

[3] Fletcher and Hopkins, 1907, *Jour. Physiol.* xxxv, 247; Harden and Maclean, 1911, *Jour. Physiol.*, xliii, 34; Batelli and Stern, 1914, *Biochem. Zeitschr.* lxvii, 443; Lund, E. J., 1921, *Amer. Jour. Physiol.*, lvii, 336. See also discussion in R. S. Lillie's book, "Protoplasmic Action and Nervous Action," Chicago, 1923, page 52, *et seq.*

which Warburg uses in support of his general theory is not a general but a particular case. The peculiar structure of the cell has thus been shown to be of great importance in its energetics. Warburg himself years ago[4] demonstrated the importance of the cell boundary in regulating oxidative metabolism when he showed that dilute sodium hydroxide accelerates the oxygen consumption of the sea urchin egg without entering the cell interior.

According to Warburg, anesthetics decrease oxidations in the cell because they are adsorbed by the iron-nitrogen, and are therefore described as general negative catalyzers of this reaction. However, there is abundant evidence[5] that dilute solutions of many anesthetics accelerate oxidative metabolism. The powerful action of the cyanides in depressing oxidative metabolism, Warburg asserts, strongly supports his theory. The validity of this evidence is open to question. Within certain limits the depression of oxidative metabolism in cyanide solutions increases with increasing concentration of the cyanide, but, after maximum depression characteristic of the concentration has been reached, continuous exposure does not result in further depression of oxidations. Warburg holds that the action of cyanide in depressing oxidations in protoplasm is due to its combination with iron, converting it into a form incapable of transferring oxygen. He regards the reaction between the cyanide and the iron as stoichiochemical, and he shows that the activity of an iron-nitrogen model in oxidizing an amino acid is depressed 97

[4] Warburg, O., 1910, *Zeitschr. f. physiol. Chem.*, cxvi, 305.

[5] Lillie, R. S., 1916, *Biol. Bull.*, xxx, 311 and references; Buchanan, J. W., 1923, *Jour. Exper. Zool.*, xxxviii, 331 and references.

per cent. by the addition of M/1000 HNC. According to this, we should expect very complete extinction of oxygen metabolism in the presence of strong cyanide. The expectation is not realized. It has been shown that M/1000 and M/2000 KNC have approximately the same effect on oxygen consumption in Planaria dorotocephala, and although M/1000 KNC is lethal within a few hours, there still remains an oxygen consumption of about ten per cent. of the normal to be accounted for.[6] In Planaria agilis and in certain molluscan tissues the remaining oxygen consumption after maximum depression by cyanide is twenty per cent. of the normal.[7] Suitably diluted solutions of the cyanides appear to accelerate metabolism and in one case at least relatively strong solutions are required to depress cellular respiration.[8] It is also true that an oxidative enzyme has been isolated, the activity of which is not appreciably affected by cyanide.[9] The wide discrepancy between the theory and the observed facts is obvious. Cyanide is therefore not a "specific negative catalyst" in the strict sense that Warburg's theory requires.

A slight recovery of oxidative metabolism in the presence of cyanide has sometimes been noted. This, Warburg says, is due to the oxidation of the cyanide itself by the catalytic action of the iron-containing substance. Since the cyanide is said to render the catalyst incapable of transferring oxygen, a reason-

[6] Hyman, L. H., 1919, *Amer. Jour. Physiol.*, xlviii, 340.

[7] Allen, G. D., 1919, *Amer. Jour. Physiol.*, xlviii, 93; Gray, J., 1924, Proc. Roy. Soc., Ser. B, xcv, 95.

[8] Hyman, L. H., 1919, *Amer. Jour. Physiol.*, xlviii, 340; Townsend, 1901, Md. Agri. Exper. Sta. Bull., No. 75, 183; Lund, E. J., 1918, *Amer. Jour. Physiol.*, xiv, 365; 1921, *ibid.*, lvii, 336.

[9] Dixon and Thurlow, 1925, *Biochem. Jour.*, xix, 672.

able doubt arises that a catalyst can be instrumental in oxidizing a substance that has rendered it incapable of catalyzing an oxidative reaction.

The writer has recently studied the recovery period after depression by KNC more intimately than has been done heretofore.[10] When Planaria are removed from a solution of KNC in which their oxygen consumption has been depressed fifty per cent., the oxygen consumption rises above the normal during the first hour after removal from the cyanide. The extent of rise above normal is considerable, is independent of the duration of depression of KNC and persists, with gradual decrement, for at least six hours. To bring Warburg's theory into alignment with these facts it is necessary to assume that the quantity of active iron or of oxidizable materials in the cell increases during the period of depression. However, the fact that the extent of rise in rate of oxygen consumption above the normal is independent of the duration of depression renders these assumptions exceedingly improbable. A third assumption is possible, namely, that the catalyst, after being freed from the cyanide, becomes excessively active. Concerning this possibility there is no information available.

Why anhydrous conditions are necessary for the action of the iron catalyst in commercial nitrogen-fixing processes or why the reaction is strongly inhibited by minute quantities of carbon monoxide and other impurities is unknown, despite the vast amount of research that has been expended. The oxidative reactions in the living organism go on in a much more complicated system. Warburg's emphatic postulation regarding the action of cyanide on the system may be considered premature.

[10] Buchanan, J. W., 1926, *Jour. Exper. Zool.*, xliv, 285.

From the effects of cyanides on oxidation in living systems it is perfectly clear that the resemblance between Warburg's models and the oxidative mechanisms in the cell is distinctly limited. As Warburg states, the idea that iron is of importance in oxidative metabolism is not new. Warburg's results have yielded suggestions as to the possible nature of the rôle of iron. However, the universality of iron in biological oxidative mechanisms is not proven, and his theory as at present formulated is quite inadequate to explain many of the facts that are associated with changes in rate of oxidations in the living organism.

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CHANGES IN THE ADIPOSE TISSUE OF THE GUINEA PIG TESTIS FOLLOWING EXPERIMENTAL TUBERCULOSIS*

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In a previous communication [1] evidence has been given to show that the very abundant formation of fibrous tissue, which occurs in the guinea pig testis following experimental tuberculosis, has its origin in an exudate present in the intertubular areas. Studies on the testicular tissues, which have been infected for various periods, show a striking and consistent series of changes, which can only be interpreted as showing the formation of the fibrillar material by a direct transformation of the exudate. The development of the fibrous tissue under such conditions appears, therefore, to be of the same nature as that which takes place during the formation of connective tissue in the vertebrate embryo.[2]

In connection with the studies as noted above, considerable attention has also been given to an examination of the infected adipose tissue, a considerable area of which lies in certain regions outside the testis, but in close contact with it and the epididymis. The present studies show that the adipose tissue following tubercular infection may also undergo a radical transformation which finally results in the formation from it of a fibrous tissue of apparently the same type as arises in the body of the testis. We may now note briefly certain changes occurring in the infected adipose tissues which result in their fibrosis.

The first histological change noted is a gradual decrease in the size of the vesicles of the fat cells which continues until in the later stages they are completely obliterated, and the adipose tissue becomes transformed into a compact fibrous tissue. The evidence appears clear that these transformation processes depend primarily upon the presence of an intercellular exudate which is either formed *in situ* in the tiny intercellular areas or else flows into them from nearby regions. As the exudate increases in amount, the areas between the fat cells are gradually enlarged and the vesicles themselves correspondingly decreased until, in the final

* From the Osborn Zoölogical Laboratory, Yale University. Work done under a grant from the Medical Research Committee of the National Tuberculosis Association.

stages, the latter have completely disappeared, and a continuous area of the exudate is present.

In the early stages the infiltrating exudate appears in the sections as either homogeneous or slightly fibrillar in character, but in the more advanced areas a marked fibrillation takes place, and the exudate becomes finally almost completely transformed into a dense fibrous tissue throughout the infected area. Unquestionably the fibrous tissue, thus formed from the infected adipose tissue, arises by the direct transformation of an intercellular material. In many cases, in cell-free areas, all stages in the process of fibrosis can be traced from the very fine fibrils up to the heavy bundles of wavy fibers which are characteristic of the fully formed fibrous tissue. Typical tubercles also gradually develop in the infected areas of the adipose tissue, and these are encapsulated and infiltrated by the newly formed fibrous tissue. In general, it may be said, as will be shown in the complete paper, that the structure of the adipose tissue is such that it affords a very favorable material for a study of the development both of the fibrous tissue and the tubercles.

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THE APPLICATION OF EXPERIMENTAL METHODS TO THE STUDY OF DEVELOPING FUNDULUS EMBRYOS

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Communicated August 26, 1927

Although the avenue of attack upon the developmental morphology of the developing Fundulus eggs was opened years ago by Morgan,[1] very little work of this nature has been completed. The outstanding contributions, in which Fundulus embryos have been used are those of Loeb along directly physiological lines, and those of Stockard[2] which have combined the morphological and physiological aspects.

During the present season it has been possible to obtain a liberal supply of Fundulus eggs and an attempt has been made to apply experimental methods to this form. The Fundulus adults were secured by netting in the waters just adjacent to the laboratory. The females were then stripped of their eggs which were fertilized with ripe sperm. The eggs were then permitted to develop at both normal and slightly subnormal temperatures.

The developmental rate, of course, varies with the temperature. Too great a retardation of development is accompanied by irregularities which give rise to abnormal embryos (Kellicott[3]). Nevertheless, the keeping of the eggs at a temperature slightly lower than normal is sufficient to retard development to a marked degree and to produce somewhat striking changes in the immediate environment of the embryos.

The eggs were permitted to develop through the segmentation stages as it was found early in the work that removal of the embryo from the eggshell during these stages frequently causes death. When the embryo has formed a distinct cap upon the surface of the yolk, it is perfectly possible to remove it from the surrounding shell and to have it continue development at a normal rate. In several cases, the embryos were removed earlier than this but the losses are large, due to the exuding of yolk substance and the subsequent death of the embryo. The high mor-

tality in these early forms is undoubtedly due to the rupture of the covering of the yolk substance.

From the time when the embryo has attained a definite form upon the surface of the yolk, it can be removed with a fair degree of certainty as to its lack of injury during removal and also as to its viability. Experiments on stages younger than the formation of the primitive streak should be performed by removing a small bit of the shell overlying the embryo and working through this opening.

The actual technique involved in the removal of the embryo from the egg shell is as follows: A sharp pair of scissors (iridectomy) such as are used by Harrison[4] in his Amblystoma technique are employed. The point which is used for the puncture of the shell should preferably be slightly longer than the other point. The shell is punctured with the sharp point. When this puncture is properly made, neither the embryo nor the yolk are in danger of injury. A small amount of fluid which is around the embryo, escapes. The point of the scissors is retained within the egg shell. The slightest amount of pressure exerted upon the egg at this time will cause the fragmentation of the embryo or a break in the yolk sack. This must be avoided by all means if the animals are later to be used for definite experimental purposes. Due to the consistency of the shell, the edges of the shell surrounding the area of puncture stick tightly enough to the point of the scissors so that the egg can be raised from the bottom of the dish. Then, with the point of the scissors under the shell (chorion) and with the assistance of a spearpoint needle, the shell is rotated upon the blade of the scissors so that a cut can be made in the shell. The cutting of the shell is continued in the same manner, the scissor point being retained under the chorion and the shell worked up upon the blade until a complete cut has been made. This cut should divide the shell as nearly as possible into equal parts, the embryo being freed from the chorion as the final cut is made separating the two parts of the shell. The embryo is thus liberated without any considerable pressure or tension being exerted upon it.

The point of making the puncture is important. In general it was found better to start with the scissors to the right of the embryo above the large omphalomesenteric veins and then continue the cut toward the head region which in my series was directed away from the operator. Probably any location which avoids possible puncture of the yolk region would do just as well.

Embryos which are injured during their removal from the egg shell, show the effect of their injury very soon after their removal. A wound in the covering of the yolk is sufficient to cause death within a few hours. The animals are more susceptible to injuries in the yolk region than to the body of the embryo itself.

Animals removed after the stage when the neutral plate has formed develop normally, i.e., with a uniform developmental rate which is at least the equal of those eggs which have been left within the shell. Considerable change occurs in the consistency of the egg shell with the time elapsed since fertilization. The shell softens from the time of fertilization irrespective of the degree of development of the embryo. There also appears to be less intrinsic pressure in eggs which have developed slowly. For this reason embryos which have developed slowly are more easily removed from the egg shell than are those which have developed faster.

After the removal of the embryos from the heavy egg shell, they may be placed in sea water, fresh water or distilled water in which they will develop. It was thought best to keep them in sea water but the other types of environment seemed to work about equally well. They will live and develop under any of these conditions provided the yolk membrane has not been injured.

After a short period during which any injury to the embryo during its removal from the egg shell may be determined, the embryos are ready for the type of operation desired. Since the embryos are small and must be handled under the microscope, a technique was devised whereby the small bits of tissue which it is necessary to remove might be easily handled. For this work, a modification of Spemann's[5] technique was used. A very fine pipette was used to hold the tissue which is to be excised and a sharp spearpoint needle was used to cut away the material. The cut was made by drawing the tissue within the lumen of the pipette and then carefully cutting the tissue with the needle against the point of the pipette. Since the embryos lack movement, no anaesthesia is required. The one point of caution in operative procedure is that the embryo will not accommodate itself to any great degree of pressure. The experimental method must provide for keeping the animals from pressure against the bottom of the dish.

With this method various results have been obtained. The importance of using the embryo freed from the shell for physiological work will form the basis of a separate communication by Dr. J. H. Bodine. The methods have been applied to problems in experimental morphology involving the response of the embryonic tissues after the extirpation of the fin; the extirpation of sense organs; the extirpation of portions of the nervous system. These studies will form the basis of separate communications.

The results of these various types of experiment are extremely interesting from the standpoint of the regenerative capabilities of this form. The preliminary results indicate clearly that there is a very high degree of determination in the tissues of the Fundulus embryos at a very early period of their development. This is indicated by the fact that there is

no regeneration of parts in embryos operated after the definite organ systems are formed.

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THE SPATIAL RELATIONS BETWEEN DEVELOPING STRUCTURES

I. THE POSITION OF THE MOUTH IN REGENERATING PIECES OF PHAGOCATA GRACILIS (LEIDY)

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THREE FIGURES

INTRODUCTION

The adult is the product of metabolic processes occurring in orderly sequence in time and space. The orderly nature of the chronology of development lends itself readily to certain types of experimentation, the results of which enable the investigator to express development in terms of velocity. A comparison of the resultant equations with the equations of non-living models is therefore possible, and similarities of these equations justify hopeful interpretations of development in terms of chemical and physical laws. The spatial relations of parts are of at least equal importance in the orderly and normal progress of development, but unfortunately non-living models that simulate these spatial relations with any degree of exactness are not at hand. Consequently, the problem of interpreting the spatial relations between developing parts in terms of quantitative laws operative in non-living systems is much less developed. The present paper is the first of a series of studies on the conditions controlling the spatial relations appearing in the development of a new individual from an isolated fragment.

THE PROBLEM

The starting-point of this investigation is the fact, first observed by Morgan ('00), that, in the regeneration of pieces of certain species of fresh-water Planaria, the site of the mouth of the regenerate has a definite relation to the original position of the piece in the parent organism. Pieces taken from the preoral region develop a mouth near their posterior ends, while pieces from the postoral region develop a mouth near their anterior ends. Although at the time Morgan suggested several explanations to account for some of the facts of regeneration in Planaria, he made no attempt to account for differences in position of the mouth in pieces from different regions. Later, the problem was further investigated by Child ('06, '12), whose conclusions will be discussed in a later paper.

In pieces of the polypharyngeal flatworm, Phagocata gracilis, this difference in location of the mouth between regenerates derived from different regions of the intact animal is very distinct. If one cuts off and discards the head of an animal of moderate size, 11 to 13 mm. long, and cuts the remainder into approximate fifths (fig. 1), after two weeks heads and tails are regenerated in all pieces except a few of those taken from the region *D* in figure 1, and in about 25 per cent of the pieces from the extreme posterior end, figure 1, *E*. The new individuals move about and feed freely. If at this time the distance from the extreme tip of the head of a regenerate derived from the most anterior fifth (fig. 1, *A*) to the approximate center of the mouth is measured (hereafter designated as the distance M) and compared with the total length of the regenerated animal (hereafter designated as the distance L), the ratio M/L is in the vicinity of 0.77. In regenerates derived from the second fifth, *B* in figure 1, M/L is 0.67. In regenerates from *C* in figure 1 M/L is 0.61; from *D*, 0.43, and in the relatively few pieces from *E* that regenerate heads within two weeks the ratio M/L is 0.32. As will be shown later, these ratios vary under different conditions.

The mouth in Phagocata gracilis is a circular aperture opening from the atrium ventrally, through which the pharynges are extruded. Its position with reference to the atrium varies; however, differences in position of the mouth are always accompanied by similar differences in the position of the atrium. The mouth is employed in this investigation as a landmark, indicating differences in position of the atrium

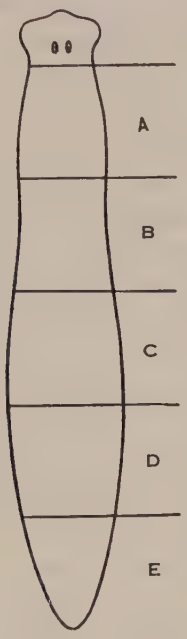

Fig. 1 Outline drawing of Phagocata gracilis, showing regions isolated in dividing into fifths.

and pharynges under the conditions of the observations and experiments. The relative position of the mouth varies in regenerates of the same length derived from the same region of animals of the same size and physiological condition. Mass experiments were therefore resorted to.

METHODS

In order to measure the animals with accuracy, it was found necessary to anesthetize them until perfectly inert. The

actual procedure of cutting and measuring the animals was as follows: From stocks that had been standardized as nearly as possible by maintenance under the same conditions in the laboratory for several weeks and feeding at regular intervals, carefully selected lots of animals of approximately the same length were taken and cut into the desired fractions, the heads being removed and discarded. The most anterior fractions were placed in an Erlenmeyer flask, capacity 500 cc., the next anterior pieces in a second flask, and so on. The flasks were set aside on the same shelf out of direct light and the water over the pieces changed at least once every forty-eight hours. At the end of the desired period, the regenerates were measured. They were first placed in strong chloretone solution for fifteen to thirty minutes, then removed singly with a pipette and placed on a stage micrometer, the scale of which was divided into tenth millimeters. The excess liquid was drawn off and the animal spread out along the scale under a binocular microscope. The total length of the animal, L, and the distance from the tip of the head to the center of the mouth, M, were recorded. Only measurements of animals with normal heads were included in the records. The animal was then washed off in a bowl of fresh water and kept for further observation and measurement or discarded, as the nature of the particular experiment required.

To accomplish the degree of anesthesia necessary to relax the animals so that they may be measured with accuracy requires a relatively brief exposure to a strong concentration of the anesthetic. It was found that the time required to handle twenty individuals was suitable for profound anesthesia without incurring losses from overexposure. Hence the results stated here for any particular experiment are based on the average measurements of twenty individuals cut, reared, and measured under as nearly as possible identical conditions, or, in the case of animals from nature, twenty individuals of approximately the same length collected at the same time and anesthetized and measured under identical conditions.

The degree of accuracy of the results obtained by these methods is indicated by the calculations of the standard deviations in twelve experiments taken at random from data comprising about 500 determinations of the mean of twenty animals. The calculations are, in terms of millimeters:

Experiment	*Mean length*	*Standard deviation*	*Mean distance head to mouth*	*Standard deviation*
1	8.93±0.049	0.324±0.034	5.74±0.046	0.304±0.032
2	3.03±0.038	0.252±0.026	2.35±0.036	0.243±0.025
3	2.99±0.037	0.246±0.026	1.63±0.024	0.164±0.017
4	8.88±0.082	0.543±0.057	4.86±0.047	0.313±0.033
5	3.61±0.028	0.184±0.019	2.65±0.019	0.129±0.013
6	3.28±0.017	0.115±0.012	1.51±0.004	0.028±0.003
7	4.02±0.032	0.217±0.023	2.74±0.018	0.123±0.013
8	3.96±0.026	0.176±0.018	2.52±0.0178	0.117±0.012
9	4.27±0.028	0.187±0.020	3.21±0.026	0.176±0.018
10	4.18±0.021	0.140±0.014	1.95±0.004	0.026±0.002
11	5.06±0.016	0.110±0.011	3.35±0.003	0.025±0.002
12	4.85±0.027	0.178±0.002	3.01±0.020	0.134±0.014

The relatively small standard deviations show that the individual variations are within comparatively narrow limits and indicate that the ratio M/L so obtained is a substantially accurate representation of the position of the mouth in the long axis of the animal.

POSITION OF THE MOUTH IN NORMAL ANIMALS

Measurements of freshly collected animals show that the ratio M/L varies inversely with the length of the animal, being somewhat greater in small animals than in large. Figure 2, line *N*, shows the location of the mouth in relation to length of the animal within the size limits of animals collected in the vicinity of this laboratory. The more anterior position of the mouth in long animals is undoubtedly due to the greater growth of the postoral region as compared with the growth of the preoral region, as the total length of the animal increases. The fact that the line *N* is straight indicates that the excess growth rate of the postoral region does not fluctuate as the length of the animal increases.

POSITION OF THE MOUTH IN REGENERATED PIECES

Figure 3 gives the results of a series of experiments which show the position of the mouth in one-half, one-third, one-fourth, and one-fifth pieces during the third week of regeneration. My records include five or more additional experiments

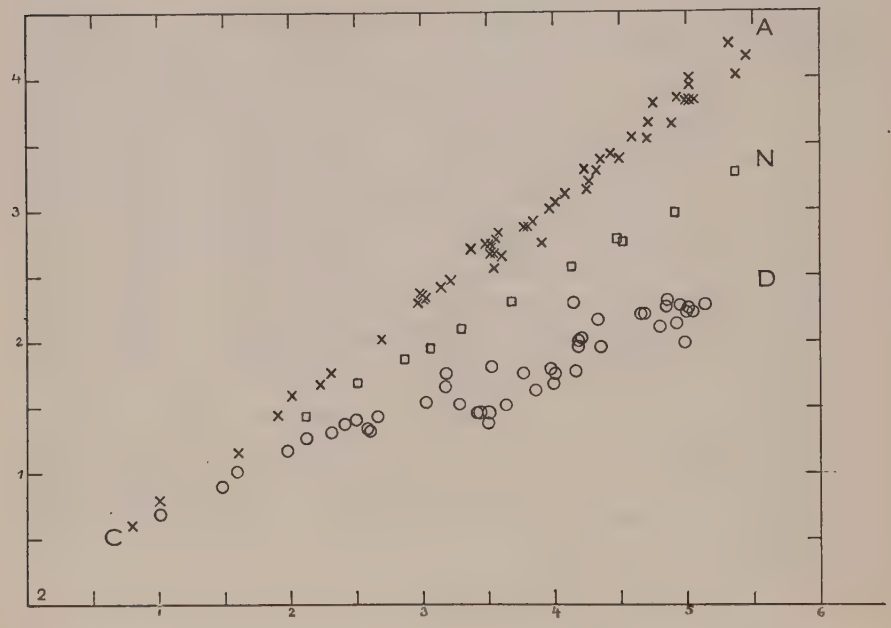

Fig. 2 Graphs showing the relation between length of the animal and position of the mouth. Ordinates are the distance from the tip of the head to the mouth, referred to as M in the text. Abscissae are the length of the animal, L. Each point represents the average of the measurement of twenty individuals. Data represented by line *A* are measurements of regenerating A $\frac{1}{5}$ pieces during the third week of regeneration; line *D*, of regenerating D $\frac{1}{5}$ pieces; line *N*, of intact animals freshly collected. Data in line *N* are plotted on half the scale of lines A and D.

of each sort giving substantially the same results. The data show that the ratio M/L of the A $\frac{1}{2}$, A $\frac{1}{3}$, A $\frac{1}{4}$, and A $\frac{1}{5}$ pieces is not appreciably different, and that the ratio M/L in pieces taken from the posterior half of the animal which include the extreme posterior end is likewise almost identical, and less than in pieces taken from this region which do not include

the extreme posterior end. From these facts it is plain that the site of origin of the new mouth in pieces from different regions cannot be regarded as a predetermined series of fixed points in the whole animal prior to cutting. Reference to these data will be made again further on.

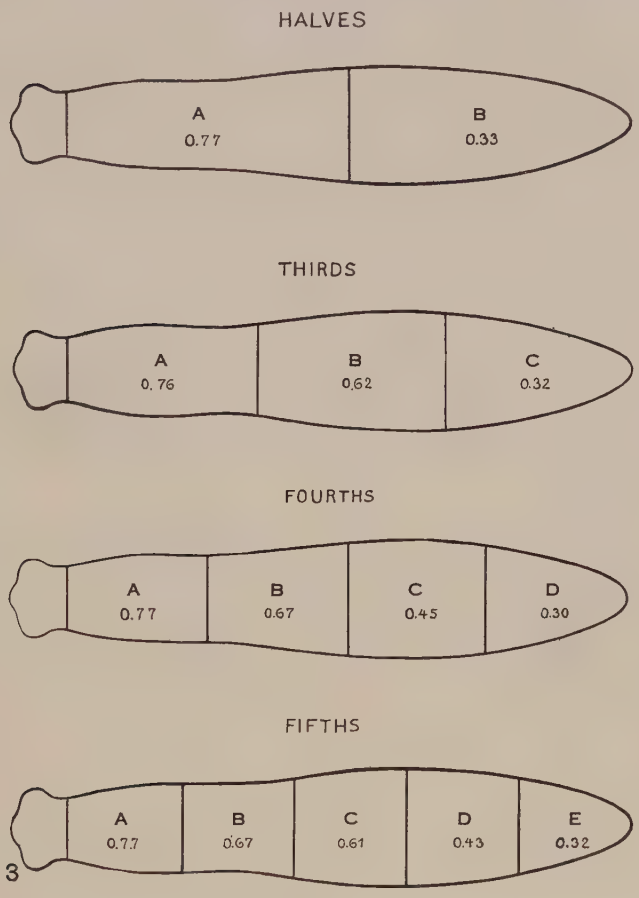

Fig. 3 Scheme showing the value of M/L of various fractions of Phagocata gracilis during the third week of regeneration. Except as noted in the text, the heads are discarded.

POSITION OF THE MOUTH IN ONE-FIFTH PIECES

For purposes of an intensive study of the position of the mouth in regenerating pieces I selected the most anterior fifth, and the fourth fifth, figure 1, *A* and *D*, for the following reasons: The viability and the head frequency of one-fifth pieces are much higher and they can be cut with more accuracy than smaller fractions; the difference in position of the mouth between A $\frac{1}{5}$ and D $\frac{1}{5}$ pieces is of the order of 77/43 (fig. 3), which is greater than the usual differences found in regenerating one-third or one-fourth pieces, which do not include the extreme posterior end of the animal, and much greater than the extreme variation found among pieces from either region. D pieces were selected instead of E pieces; in the former head frequency is much higher; furthermore, D pieces have two cut surfaces, hence physiological conditions due to cutting are more comparable in A and D pieces than in A and E pieces.

An extended series of experiments was carried out to determine if the value of M/L in A $\frac{1}{5}$ and D $\frac{1}{5}$ pieces from large animals is different from the value of M/L in such pieces taken from small animals. The results are given in figure 2, line *A* and line *D*. From these results it is evident that, in the case of A $\frac{1}{5}$ pieces, M/L is fairly constant and independent of the length of the animal from which the piece is taken. This is not true of D $\frac{1}{5}$ pieces, for the line *D* in figure 2 approaches the line *A* and apparently coincides with it at approximately the point *C*. Hence it is shown that as D $\frac{1}{5}$ pieces are taken from smaller and smaller animals the value M/L becomes greater and greater. In other words, in D $\frac{1}{5}$ pieces cut from small animals the new mouth appears more posteriorly than in D $\frac{1}{5}$ pieces cut from large animals.

The data thus far presented are measurements taken during the third week of regeneration. The next step was to determine whether the value of M/L in a regenerating piece is constant after this time. This was accomplished as follows: Four hundred animals were selected on the basis of similarity of state of nutrition, size, and appearance, and divided into

two lots of 200 each. A $\frac{1}{5}$ and D $\frac{1}{5}$ pieces were cut from each lot and allowed to regenerate, the A and D pieces of each lot being kept in separate flasks. During the third week of regeneration twenty individuals of each type from both lots were measured and returned to their respective flasks. Thereafter the A and D pieces of one lot were fed twice a week on fresh beef liver, while the pieces in the other lot were kept without food. From time to time twenty individuals from both types of fed and starved regenerates were measured and returned to the flasks. Since occasional losses were unavoidable, the data given do not represent measurements of the same twenty animals in each flask throughout the duration of the experiment, but of the longest twenty at the times of measurement. The experiment was continued over four months.

The results are given in table 1, and show: 1) that the value of M/L in A $\frac{1}{5}$ pieces when fed decreases as the length of the animal increases, approaching the value of M/L of normal animals, as shown in line *N*, figure 2. 2) In starving A $\frac{1}{5}$ pieces the value of M/L does not change with decrease in length of the piece. The decrease in length of A $\frac{1}{5}$ pieces during four months of starvation is relatively very small. 3) In fed D $\frac{1}{5}$ pieces M/L increases in value as the length of the regenerates increases. 4) In starving D $\frac{1}{5}$ pieces M/L also increases in value, but the total length of the pieces decreases slightly. This experiment was repeated on a somewhat lesser scale with substantially the same results as shown in table 1.

These data merely confirm and extend the facts already observed by Morgan ('00) and Child ('12). Because of the distinct differences shown, they merit further comment. In fed A $\frac{1}{5}$ pieces M/L changes, the mouth becoming relatively more anterior. In starved A pieces there is no change in the value of M/L. In starved D pieces M/L changes, the mouth becoming relatively more posterior. In fed D pieces also the mouth becomes relatively more posterior. When considered together, these facts indicate that there are distinct

differences in the processes of regulation between pieces from different regions. In fed A pieces growth is more rapid posterior than anterior to the mouth, for the results in the starved A pieces indicate that no internal rearrangement of

TABLE 1

Changes in relative position of the mouth in regenerating A $\frac{1}{8}$ and D $\frac{1}{8}$ pieces during feeding and starvation. Cut five days after feeding, on November 28th

	L	M	M/L
		A fed	
December 12th	3.57	2.81	0.78
December 30th	6.47	4.48	0.69
January 13th	7.88	5.25	0.66
February 1st	8.93	5.74	0.64
February 28th	8.69	5.42	0.62[1]
		D fed	
December 12th	3.98	1.78	0.44
December 30th	6.63	3.44	0.51
January 13th	8.19	4.39	0.53
February 1st	8.88	4.86	0.55
February 28th	9.37	5.18	0.55
		A starved	
December 12th	3.48	2.73	0.78
December 30th	3.40	2.65	0.78
January 13th	3.37	2.59	0.77
February 1st	3.03	2.35	0.78
February 28th	2.19	1.68	0.76
		D starved	
December 12th	4.03	1.75	0.43
December 30th	3.64	1.74	0.47
January 13th	3.27	1.68	0.51
February 1st	2.99	1.63	0.54
February 28th	2.10	1.20	0.57

[1]A number of the longest individuals in this lot were lost on February 1st; this accounts for the decrease in the value of L on February 28th.

materials occurs. Regulation in the position of the mouth in A pieces is therefore effected solely by the fact that regions posterior to the mouth utilize foods more rapidly with a consequently greater increment in growth in the post- than in the preoral region. The change in the value of M/L in A pieces is therefore accomplished in the same manner as in the growth and increase in length in the normal animal, as shown in figure 2, line *N*.

In starved D pieces regulation in the position of the mouth is effected apparently by the transfer of materials from the post- to the preoral region—a process described by Morgan as morphallaxis. Since regulation is not effected in this manner in A pieces, it is evident that morphallaxis takes place only from posterior to anterior. In other words, the preoral region in D pieces is maintained at the expense of the postoral region. In fed D pieces a similar change of relative position of the mouth occurs, but whether it is largely by morphallaxis or by a more rapid growth of the preoral region it is at present not possible to say. In either event, it is strongly indicated that metabolic activity is greater in the pre- than in the postoral region of D pieces during the period of regeneration covered by this experiment.

Both the A $\frac{1}{5}$ and the D $\frac{1}{5}$ pieces are totipotent, but the processes whereby the form of the normal animal is attained by each differ. In starved D pieces the process is carried out by the utilization of materials present in the fragment. Presumably, the same materials are present in much the same amounts in A pieces, yet regulation is not effected in the absence of an external food supply sufficient to permit growth. The obvious explanation is that the differences are due to the position of the regions of greatest metabolic activity with reference to the materials available in the piece. If this is true, in D pieces the greater metabolic activity is in the preoral region and the materials necessary for the maintenance of function in this region are obtained during starvation by utilization of materials present in the less active postoral region. In A pieces the quantity of material located

posterior to the mouth is not sufficiently great and the difference in metabolic activity between the pre- and postoral regions apparently does not favor the transfer of materials anteriorly. External sources of food being afforded, the regenerate grows and develops sooner or later a posterior region of relatively rapid growth, as in the case of increase in length of a small normal animal.

Referring to figure 3, it is seen that all the anterior pieces which include the region just posterior to the head develop the new mouth at the same relative position, the ratio M/L of A pieces of all fractions being 0.76 to 0.78. Similarly, fractions cut to include the extreme posterior end, B $\frac{1}{2}$, C $\frac{1}{3}$, D $\frac{1}{4}$ and E $\frac{1}{5}$, have much the same value for M/L, 0.32. From these facts it seems probable that some sort of influence is exerted by the extreme anterior and posterior regions, or at least so much of them as is included in the A $\frac{1}{5}$ and E $\frac{1}{5}$ pieces, that in some way conditions the location of the mouth in fractions which contain these regions. If this is true, it ought to be possible to alter the relative position of the mouth in anterior pieces by leaving the head attached for a longer or shorter period during regeneration, i.e., including in the piece more of this anterior region which seems to be instrumental in controlling the position of the mouth in the regenerates. A series of experiments of this sort employing A $\frac{1}{5}$ pieces was performed, the results of which appear in table 2. While the differences between controls and pieces with heads attached for forty-eight hours or longer after section are not great, the data show consistently that the value of the ratio M/L is greater in pieces which include the head for some period of regeneration than in pieces in which the head has been removed at the beginning. That the relatively higher value of M/L in pieces with heads attached is not due to a relatively shorter postoral region resulting from less posterior regeneration is shown by the data in column 5, table 2. Of the nine cases, in five the postoral region is shorter in the pieces with heads attached, in two cases the postoral region in such pieces is longer, and in two cases there is no appreciable difference.

In the case of the posterior end, the data already given in figure 3 suggest that the presence of this region also has an influence on the location of the mouth in the regenerate. A further study of this probability was made in a series of experiments carried out with the object of finding whether or not the presence of the posterior end for a limited time during regeneration would affect the position of the mouth

TABLE 2

Position of the mouth in A $\frac{1}{5}$ pieces during third week of regeneration. A $\frac{1}{5}$, control; A $\frac{1}{5}$ with head attached forty-eight hours, three days, four days, and throughout the experiment

EXPERIMENT	TYPE OF PIECE	L	M	M/L	L-M
1	A $\frac{1}{5}$ control	3.53	2.70	0.76	0.83
	A with heads forty-eight hours	3.56	2.87	0.81	0.69
2	A $\frac{1}{5}$ control	2.99	2.30	0.76	0.69
	A with heads throughout	3.45	2.81	0.81	0.64
3	A $\frac{1}{5}$ control	3.78	2.87	0.75	0.91
	A with heads throughout	3.99	3.20	0.80	0.79
4	A $\frac{1}{5}$ control	4.36	3.39	0.75	0.97
	A with heads four days	4.85	3.83	0.79	1.02
	A with heads throughout	5.10	4.01	0.80	1.09
5	A $\frac{1}{5}$ control	3.55	2.55	0.71	1.00
	A with heads three days	3.23	2.53	0.80	0.70
	A with heads throughout	3.58	2.83	0.79	0.75
6	A $\frac{1}{5}$ control	3.22	2.47	0.76	0.75
	A with heads forty-eight hours	3.09	2.50	0.80	0.59
	A with heads throughout	3.36	2.64	0.78	0.72

of the regenerates. D $\frac{1}{5}$ pieces were cut to include the E $\frac{1}{5}$ region, i.e., the posterior two-fifths piece (fig. 1); other D $\frac{1}{5}$ pieces were cut as controls, and in the D plus E pieces the E region was removed from D after certain periods of regeneration. After two weeks the regenerates were measured in the usual way. The results are given in table 3. Without exception, they show that in D pieces with E attached for some period the value of M/L is higher than in controls. In

some cases the difference is slight; in others, quite marked. The presence of the E region in some way causes the new mouth to appear farther posterior. These data also show

TABLE 3

Position of the mouth in D $\frac{1}{8}$ pieces. D $\frac{1}{8}$ control; D $\frac{1}{8}$ with the E $\frac{1}{8}$ region attached for twenty-four hours, forty-eight hours, three days, four days, five days, and duration of experiment. Measured during the third week of regeneration

EXPERIMENT	TYPE OF PIECE	L	M	M/L
1	D $\frac{1}{8}$ control	4.85	2.31	0.47
	D with E attached twenty-four hours	4.38	2.43	0.55
2	D $\frac{1}{8}$ control	3.42	1.45	0.42
	D with E attached twenty-four hours	3.20	1.73	0.54
3	D $\frac{1}{8}$ control	5.03	2.23	0.44
	D with E attached forty-eight hours	4.90	2.59	0.52
4	D $\frac{1}{8}$ control	4.00	1.66	0.40
	D with E attached twenty-four hours	3.61	1.56	0.43
	D with E attached forty-eight hours	3.66	1.75	0.47
5	D $\frac{1}{8}$ control	4.66	2.20	0.47
	D with E attached twenty-four hours	5.02	2.51	0.50
	D with E attached forty-eight hours	4.91	2.45	0.50
6	D $\frac{1}{8}$ control	4.80	2.10	0.43
	D with E attached four days	5.00	2.28	0.45
7	D $\frac{1}{8}$ control	4.00	1.66	0.40
	D with E attached five days	3.66	1.74	0.47
	D with E attached five days	3.27	1.48	0.45
8	D $\frac{1}{8}$ control	3.44	1.46	0.42
	D with E attached three days	3.53	1.52	0.43
	D with E attached throughout experiment	4.70	1.44	0.30
9	D $\frac{1}{8}$ control	3.51	1.45	0.41
	D with E attached forty-eight hours	3.50	1.47	0.42
	D with E attached throughout experiment	5.15	1.61	0.31

that the position of the new mouth is partially determined during the first twenty-four hours after section. Reference to table 2 shows that this is also true in the case of anterior pieces.

Summing up, the results of measurements show: 1) The relative position of the mouth in animals from nature varies with the size of the animal, becoming more anterior as the length of the animal increases. 2) When cut into pieces, the mouth in the regenerates appears more anteriorly as the region from which the piece is taken becomes more posterior. 3) Pieces cut from the region immediately posterior to the head develop a new mouth at the same relative position in fractions ranging from one-half to one-fifth the total length. Pieces taken to include the extreme posterior end of the animal show a similar characteristic. 4) The relative position of the mouth is constant in pieces, approximate fifths, taken just posterior to the head, regardless of the length of the animals from which the pieces are taken. In pieces taken from the posterior region, approximately the fourth fifth, the relative position of the mouth varies with the length of the parent animal, being more posterior in pieces taken from small than in pieces from large animals. 5) When pieces taken just posterior to the head are fed and grow, the position of the mouth changes, becoming more anterior as the length of the regenerate increases, approaching the relative position of the mouth in animals of corresponding size in nature. No marked change in position of the mouth results when pieces from this region are starved for protracted periods. 6) When pieces from the posterior region are fed, the mouth becomes relatively more posterior as the length of the regenerate increases, approaching the condition found in animals of corresponding length from nature. In starved pieces from this region in which the total length decreases with advancing starvation, the mouth also becomes more and more posterior, the changes in its relative position being essentially like those in fed pieces. 7) Anterior pieces which include the head of the parent animal develop the new mouth at a relatively more posterior position than in such pieces in which the head is removed at the time of isolation. Posterior pieces cut to include the extreme posterior region of the animal for a limited period after isolation develop the

new mouth at a relatively more posterior position than do such pieces in which the extreme posterior end is removed at the time of isolation.

DISCUSSION

These remarkable facts demand explanation. Obviously, the reasons for the differences in position of the mouth between pieces from different regions must be sought in either structural or physiological conditions in pieces from various regions, and in differences in these regions in large and small animals. The first to be considered are the structural differences between regions which, when isolated, show differences or similarities in the value of M/L.

Only two fairly complete accounts of the structure of Phagocata gracilis are found in the literature, namely, those of Woodworth ('91) and of Peaslee ('10), of which the former is more detailed. From these descriptions and my own observations it may be stated that A $\frac{1}{5}$ and D $\frac{1}{5}$ pieces, which exhibit striking differences in the location of the mouth, have in common the integumentary layers and associated tissues, parenchyma, portions of the testes and vasa deferentia, portions of the alimentary and nervous systems, portions of the excretory canals, and muscle tissue. The ovaries and vitellaria are found in the A $\frac{1}{5}$ pieces, but not in the D $\frac{1}{5}$ pieces. A pieces contain a portion of the single anterior branch of the gut; D pieces contain portions of the two posterior branches. D pieces also contain a portion of the seminal receptacle and associated structures. Both A and D pieces contain a small portion of the atrium and pharynges.

Is the site of origin of the new mouth dependent on the location and amount in the piece of the pharyngeal material of the intact animal? According to Woodworth, the atrium is one-half the length of the animal and is located midway between the anterior and posterior ends. My own observations indicate that his description is substantially correct in the case of animals of moderate size. Remembering that the head is cut off and discarded (a piece between 1 and 2

mm. long in the case of animals of moderate size), the posterior half of A pieces and the anterior half of D pieces contain, respectively, anterior and posterior portions, approximate fourths, of the atrium and pharynges. If the position of the new mouth were related to the amount of pharyngeal or atrial material in the piece, one would expect closely similar values of M/L in A $\frac{1}{5}$ and D $\frac{1}{5}$ pieces—an expectation which is not realized. If the position of the new mouth were related to the position of the atrial or pharyngeal material in the piece, one would expect that the distance from the mouth to the posterior end in A $\frac{1}{5}$ pieces should be equal, or nearly equal, to M in D $\frac{1}{5}$ pieces, since A contains the anterior fourth and D the posterior fourth of the atrium, oppositely situated. This expectation is also not realized; M/L in A pieces is 0.77 and in D pieces 0.43. Morgan and Schiedt ('04) state that in pieces taken anterior to the atrium the new pharynges develop in the posterior region of the pieces, while in posterior pieces containing none of the old pharyngeal material, new pharynges develop in the anterior region. Furthermore, in one-fourth pieces (fig. 3), A $\frac{1}{4}$, and in one-half pieces, A $\frac{1}{2}$, have essentially the same value for M/L, yet the A $\frac{1}{4}$ piece contains slightly more than one-fourth, while the A $\frac{1}{2}$ piece contains slightly more than one-half the old atrium. From these facts and others discussed farther on, it is plain that the amount of old pharyngeal or atrial material present and its location in the piece has no important relation to the site of origin of the new mouth.

Similarly, there is no relation apparent between the presence of nervous or other specific tissue and the position of the mouth in the regenerate. The data in figure 3 show that in A $\frac{1}{5}$, A $\frac{1}{4}$, A$\frac{1}{3}$, and A $\frac{1}{2}$ pieces there is no appreciable difference in the value of M/L, yet all these fractions contain different amounts of the same specific tissues. It may also be stated that the animals of the same size yield the same general results as regards the relative position of the mouth in regenerating pieces, regardless of the season of the year and the stage of sexual maturity at the time of cutting.

Some emphasis has been placed on the rôle of certain neoblast or formative cells in regeneration. According to Curtis and his students (Isely, '24), these cells in Phagocata gracilis are most numerous in the anterior region of the animal, decreasing in number posteriorly. Sections of my material stained with Wright's triple stain show cells which I judge to be identical with Curtis' formative cells present in both A $\frac{1}{5}$ and D $\frac{1}{5}$ pieces. Although no counts were made, they appear to be somewhat more numerous in the A than in the D pieces. Undoubtedly, formative cells enter into the processes of regeneration, but I am unable to conclude that there is any association between the number of these elements present in the piece and the relative position of the mouth and associated structures in the regenerates. For instance, in figure 3 it is shown that the A $\frac{1}{2}$, A $\frac{1}{3}$, A $\frac{1}{4}$, and A $\frac{1}{5}$ pieces develop the new mouth at the same relative position with no relation to probable differences in the number of formative cells apparent. Likewise in B $\frac{1}{2}$, C $\frac{1}{3}$, D $\frac{1}{4}$, and E $\frac{1}{5}$ pieces an association between the number and distribution of the formative cells present and the position of the new mouth is not evident.

The data in figure 3 also indicate that the position of the region from which a piece is taken with reference to the mouth of the intact animal is of no significance in determining the position of the mouth in the regenerate. In one-fifth pieces taken from animals of moderate size the old mouth falls in the C piece; yet in A $\frac{1}{2}$, A $\frac{1}{3}$, A $\frac{1}{4}$, and A $\frac{1}{5}$ pieces the new mouth appears at approximately the same relative position. Summing up, differences in the distribution of specific tissues or of specific cells do not appear to account for differences in the position of the new mouth in regenerating pieces taken from different regions of the animal.

The theory of Sachs, in recent years develop as an application of the law of mass action and vigorously defended by Loeb, that regeneration depends on the concentration of some specific substance or substances, is not adequate to explain the experimental facts presented here. A serious objection

to this conception arises from the fact that there is no evidence that any of the branches of the digestive system, or of the excretory system, intercellular spaces, and pseudocoele in flatworms, nor in the various elements of circulatory systems in general, are directive for any of the specific constituents of the contained fluids. If we assume that these hypothetical substances follow the laws of diffusion in an aqueous medium, their distribution must be general, the several tissues being the selective agents. Given the materials, specific end products are elaborated in a reaction system only where the requisite energy relations exist. Likewise, the specific materials involved in building up the atrium and pharynges in a piece of flatworm undergoing regeneration may be expected to be elaborated from a generalized food supply only by cells in which the requisite physiological conditions obtain. In a word, the factors which determine the regeneration of the mouth and its associated structures are to be sought in intrinsic cellular processes, not in their food supply.

Further and specific objections to the application of this interpretation here include the fact, already mentioned above, that figure 3 shows M/L to have essentially the same value in all A pieces, regardless of the fraction of the animal included. Mass of tissue, or mass of contained substances, has apparently no causal relation to the value of M/L within the size limits of the fractions employed in this investigation. Moreover, if this hypothetical substance is a food, it is not utilized by the animal during protracted starvation, for M/L in pieces cut from an animal after seven weeks of starvation is not appreciably different from M/L in pieces taken from well-fed animals. I believe good and sufficient reasons have been given here for rejecting the conception of 'formative stuffs' as an explanation of the determination of the position of the mouth in regenerating pieces of Phagocata gracilis.

From the foregoing it is apparent that the solution of the problem at hand is to be sought in the physiological rather than the structural conditions or distribution of specific materials in the organism. From the data presented, partic-

ularly those in figure 3 and table 2, these facts appear: 1) The anterior region of the animal is the locus of origin of some sort of physiological influence that plays a part in determining the location of the mouth in fragments which contain this region; 2) the nature of this influence changes when less of the anterior end is included in the piece, as in A $\frac{1}{5}$ and B $\frac{1}{5}$ pieces. The data in figure 3 and table 3 show that the removal of the extreme posterior end at the time of isolation has a marked effect on the location of the mouth in posterior pieces. These data also bring out the interesting and important fact that the mechanism which controls the location of the new mouth is operative very shortly after the piece has been isolated, undoubtedly during the first twenty-four hours.

The intact animal must be regarded as a functioning system of various types of tissues and organs. When a fragment of this system is isolated, it is not immediately converted into a new system, but retains for a longer or shorter period the tissues and functional correlations of the original system. The conversion or development of the tissues into appropriate parts of a new system is not completed in twenty-four hours; in fact, it has only begun. It is therefore plain that the factors, whatever they may be, that determine the site of origin of the new mouth in a regenerating piece operate in some manner through the medium of the differentiated tissues of the original animal from which the piece is taken.

It is apparent from the data that the value of M/L in anterior pieces is greatest when the extreme anterior region of the animal is included in the piece. From a study of figure 3 and table 3, it is seen that when the extreme posterior region is included in the piece throughout the period of regeneration, the value of M/L is less than in pieces in which the extreme posterior region is removed at the time of isolation. On the other hand, if the extreme posterior region is left attached for only a limited period after isolation, the value of M/L is thereby increased. From these results two facts appear, namely, that the presence of the extreme pos-

terior region throughout the entire two-week period of regeneration determines a relatively low value for M/L, and that the act of cutting off the extreme posterior region, if performed during the early period of regeneration, in some manner makes for a decrease in the value of M/L (table 3).

That the extreme anterior and posterior regions of the animal exert more or less control over the site of origin of the new mouth in regenerating pieces is clear. But these two regions do not constitute mouth-determining regions in the sense that they cause the development of a mouth; they merely influence some sort of system inherent in each piece that determines the site of origin of the new mouth. The rôle of the anterior region of the intact animal in determining a high value for M/L in anterior pieces decreases as the region from which the piece is taken becomes more posterior. Similarly, the rôle of the posterior region of the animal in determining a low value for M/L in posterior pieces becomes less as the region from which the piece is taken becomes more anterior.

In figure 2, line *D* shows that the posterior region of small animals is less effective in determining a low value for M/L in D $\frac{1}{5}$ pieces than in large animals. Since the line *N* in figure 2 shows that in small animals the region posterior to the mouth is less extensive than the postoral region of large animals, the inference is plain that the posterior region becomes more and more effective in determining a low value for M/L in D $\frac{1}{5}$ pieces as the length of the postoral region increases, i.e., as postoral growth increases. The development of this posterior determining region is therefore in some way associated with the growth of the posterior end of the animal.

The question then arises: What is the nature of the physiological properties of the extreme anterior and posterior regions of the animal that are the effective agents in influencing the morphological characteristics of regenerating fragments? In the case of intact normal animals, it is shown by line *N* in figure 2 that the definitive position of the mouth

as the animal passes from young to older stages is conditioned upon the relative rates of growth metabolism of the pre- and postoral regions. Similarly, the data in table 1 strongly indicate that the position of the mouth during the later period of regeneration of pieces is related to the relative rates of growth metabolism in the anterior and posterior regions of the regenerate.

As regards the factors in an isolated piece which determine the site of origin of the new mouth, apparently operative during the first twenty-four hours of regeneration, the fact that these factors are not to be ascribed primarily to structural or qualitative conditions compels one to accept the view that the position of the new mouth in a regenerating piece is an expression of a correlative mechanism that is essentially physiological and transmissive rather than morphological or transportive in character. Since the position of the mouth in regenerating pieces from various regions differs quantitatively, it follows that the mechanism of correlation in each piece is a quantitative physiological relation of some sort.

If this is correct, the problem then becomes a problem of the nature and relation of quantitative physiological conditions within a piece after isolation. In a later paper experimental facts concerning quantitative physiological conditions within a piece and differences in these conditions between pieces from various regions will be presented. A possible interpretation of the rôle played by these conditions in determining the site of origin of the new mouth is reserved for the later paper.

SUMMARY

This paper is the first of a series dealing with the factors determining the location of the mouth in the polypharyngeal flatworm, Phagocata gracilis. It includes evidence which shows:

1. That the relative position of the mouth varies with the length of the animal, being more anterior in large animals than in small.

2. In the regeneration of pieces the mouth appears more and more anteriorly as the region from which the piece is taken becomes more and more posterior.

3. In pieces cut from just posterior to the head the new mouth develops at the same relative position regardless of the fraction of the total length of the animal included in the piece.

4. Pieces cut to include the extreme posterior region of the animal, having no posterior cut surface, show a similar characteristic.

5. In pieces cut from just posterior to the head which represent the same fraction of the total length of the animal, the new mouth develops at the same relative position regardless of the length of the animal from which the piece is taken.

6. In pieces taken from the same posterior region representing the same fraction of the total length of the animal, the relative position of the new mouth varies with the length of the parent animal, being more posterior in pieces taken from small than in pieces from large animals.

7. When pieces taken from just posterior to the head are fed and grow, the relative position of the mouth changes, becoming more anterior as the regenerate increases in length. No change occurs in the position of the mouth when pieces from this region are starved for many weeks.

8. When pieces taken from a posterior region are fed, the mouth becomes relatively more posterior as the regenerates increase in length. In starved pieces from the same region the changes in position of the mouth are essentially like those in fed pieces.

9. Complete regulation of the position of the mouth in regenerating pieces requires at least four months at laboratory temperatures.

10. Anterior pieces which include the head of the parent animal develop the new mouth at a relatively more posterior position than do such pieces in which the head is removed at the time of isolation. Posterior pieces cut to include the extreme posterior region of the animal for a limited period

after isolation develop the new mouth at a relatively more posterior position than do such pieces in which the extreme posterior region is removed at the time of isolation.

11. The site of origin of the new mouth in regenerating pieces is at least partially determined during the first twenty-four hours after isolation.

It is pointed out that these facts are not to be explained on the basis of structural conditions or the distribution of specific tissues or substances. The evidence strongly indicates that the mechanism that conditions the site of origin of a new mouth in regenerating pieces is essentially physiological and quantitative in character.

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FURTHER STUDIES ON THE MORPHOGENETIC RÔLE OF THE SOMITES IN THE DEVELOPMENT OF THE NERVOUS SYSTEM OF AMPHIBIANS

THE DIFFERENTIATION AND ARRANGEMENT OF THE SPINAL GANGLIA IN PLEURODELES WALTLI

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INTRODUCTION

The student of experimental embryology who follows closely the present development of this field is impressed by the steadily increasing number of facts which often lead to the conception of the mutual relationship of the developing embryonic system. Organs which seemed to be entirely self-differentiating at first sight have proved to be influenced also by the surrounding tissue, and other organs apparently showing dependent differentiation sometimes exhibit some inherent determination. Each stage of development seems to rep-

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resent an equilibrium of many single factors which sometimes overlap in their effects, and therefore offer many difficulties to experimental analysis.

The earliest development of the nervous system in amphibians seems to be determined by the action of various factors, located partly within the medullary system, partly in the neighboring tissues. It was found by Spemann and his students that the invaginated roof of the archenteron is able to determine the medullary plate in Triton (Spemann, '18, '19; Marx, '25; Wessel, '25; Bautzmann, '26), and that even presumptive epidermis may be induced to form medullary substance by a piece of underlying roof of the archenteron (Spemann and H. Mangold, '24; Geinitz, '25; Marx, '25). On the other hand, it was shown by Goerttler ('25, '26) that in Pleurodeles the presumptive medullary material is partly determined in the early gastrula stage, before the roof of the archenteron is invaginated.

The writer (Lehmann, '26 b) was able to demonstrate that the medullary material in Triton also is determined before invagination has proceeded very far. It is clear from this that, at least in Triton, two factors, which are partly overlapping, determine the medullary plate. The stages prior to the closing of the medullary tube are influenced somewhat by the somites (Lehmann, '26 a). Distribution and position of the cells and the formation of the medullary folds are dependent on the presence of the mesoderm. The floor plate of the spinal cord fails to thin out, in case of fusion of the somites underneath the spinal cord. A decrease in mass of the somites of one side results in a similar deficiency of mass on the corresponding side of the spinal cord.

The next question to be answered was: How far are the structures in the spinal cord determined after closing of the medullary folds and how is their further development affected if the somites are removed at this stage? It seemed likely that such experiments also would throw some light on the question, how the segmental arrangement of the spinal ganglia is connected with the segmentation of the mesoderm. This

seemed to be of interest from a comparative anatomical viewpoint.

Experiments on the morphogenetic influence of the somites on the nervous system after closing of the medullary folds are not very numerous. Shorey's paper ('09) deals with this question. Her results on the influence of deficient somites on the development of the motor neuroblasts were not corroborated by Detwiler's very extensive experiments ('26 a). The abnormalities she obtained in the developing spinal ganglia aroused no further interest. The influence of the somites on the spinal ganglia was studied by Detwiler ('26 b, '27) only in so far as changes in the sensory area of the muscles affect the number of ganglion cells. But as soon as the rôle of the mesoderm in the earliest development of the nervous system of the amphibians was recognized, the investigation of this problem received a new stimulus.

METHODS

All experiments described below were carried out on the embryos of Pleurodeles waltli, a species closely related to Triton. By means of low temperature, about 10°C., the writer obtained eggs from two pairs of Pleurodeles during the latter part of July, the beginning and the end of October, 1926.

All operations were performed in Ringer's solution, which has been used in preference to plain sodium-chloride solution. Observations on wound healing and the behavior of the operated tissues in this solution proved its exceedingly favorable influence. In the series in which a large piece of medullary tube with adhering somites and notochord was grafted upon another embryo, the mortality was only 22 per cent.

The composition of this solution is as follows: NaCl, 6.5; KCl, 1.4; $CaCl_2$, 1.2; $NaHCO_3$, 0.2; NaH_2PO_4, 0.001; water, 1000.

The results of some experiments on the influence of hypotonic sea-water on the developing Arbacia eggs seem to indicate that the embryos develop much better in hypotonic sea-

water containing relatively many Ca- and OH-ions. The solution used for the operations also had a relatively high Ca and OH content, 0.12 per cent, respectively, pH of 7.8 to 8.0. The embryos operated in this medium showed a smooth wound surface and healed rapidly. Even embryos having the susceptible entoderm and mesoderm exposed for several days in fistulas survived in many cases. Mendeléeff ('25) found a similar effect of a high Ca content and low pH in tissue cultures. Under these circumstances proliferation is stimulated.

After the operation, the embryos were transferred to a solution which contained equal parts of this Ringer solution and spring water. It seemed very favorable to keep the medium of the growing animals at about pH 7.8. It was noticed that the use of spring water of pH 6.6 resulted in a higher mortality of the embryos, which in many cases was foreshadowed by the appearance of mucus, covering the whole body and especially the gills. This never occurred in alkaline solutions.

The animals were preserved in Bouin's fluid. The sections were stained with Delafield's haematoxylin and Congo red. In order to get a very uniform and bright stain with Congo red it was found necessary to use a mordant, i.e., to dip the sections after being stained with haematoxylin for a second in 70 cc. water containing 2 cc. Bouin's fluid. After that the sections were dipped in pure water, in order to remove the larger part of the adhering solution. Then the sections were placed for one-half to one minute in an aqueous solution of Congo red, washed in water, then placed in 35 per cent alcohol for two minutes, and then dehydrated, cleared, and mounted. The sections treated by this method proved to be very satisfactory. It is, of course, not possible to state how long this stain will last.

EXPERIMENTAL

a. Complete unilateral removal of several somites

In these experiments (series PES) embryos were used in stages after the closing of the medullary folds and before the first appearance of the ear vesicle. Sections show that at this

time no ganglion cells can be found between the somites and the medullary tube. Consequently, it is very unlikely that they were injured or removed by excision of the somites. This point will be discussed more fully in one of the following sections. The operation consisted in the complete removal of a piece containing three to eight somites of one side and the epidermis covering them. The cells adhering to the medullary tube and the notochord were carefully removed till one could see clearly the surface of the spinal cord and the notochord. The wound was then covered by a piece of skin taken from another embryo of the same age. In some cases skin from the belly was transplanted, in another series a limb bud was grafted into the wound.

As soon as the embryo showed the first contractions, the deficiency of the excised somites could be noticed. When stimulated by a hair, the normal side bent in a regular arc, but on the operated side only head and tail were lifted, and the defective region failed to exhibit any contraction. In later stages, however, a rapid swimming movement was established, which showed in many cases but slight irregularities (see also Detwiler, '27).[2]

Abnormalities in the pigmentation of the operated region occurred very often. In some extreme cases the skin in this region was without any melanophores and almost transparent (fig. 1) (see also Detwiler, '27). In other cases melanophores were present, but not distributed in the normal pattern. In some cases the outgrowing lateral line was stopped by the implanted piece and formed a group of sense organs near the anterior border of the graft. As a rule, it was found that the distribution of the melanophores, where the midbody line failed to grow out, differed typically from the normal picture.

[2] I am very much indebted to Dr. S. R. Detwiler for having given me the opportunity to read the manuscript of his paper: "The effect of extensive muscle loss upon the development of spinal ganglia in Amblystoma" (Jour. Exp. Zoöl., vol. 48). In his experiments three somites were excised, but purposely some muscle was left and the wound was not cleaned as thoroughly as in my cases. Therefore (see p. 109), he never found absence of spinal ganglia which is caused apparently only by complete removal of somites.

The groups of the dorsal melanophores are separated from the ventral groups by a pigmentless zone, in which the mid-body line is situated. In the case of the lack of the mid-

Fig. 1 PES 70. Killed forty-seven days after operation. After fixation. × 8. The defective region on the left side is characterized by the complete absence of melanophores.

Fig. 2 PTM 23. Killed sixty-five days after operation. After fixation. × 8. The graft containing spinal cord, somites, and notochord is situated on the left flank and shows a well-developed dorsal fin.

body line, this pigmentless zone is crossed by many groups of melanophores connecting the dorsal and the ventral groups. This observation seems to indicate that there exist some relations between the arrangement of the melanophores of the

body wall and the presence of the midbody line of the lateral-line organs.

The general form of the body was often very little changed by these defects, the zone of defect being merely indicated by the different pigmentation.

b. Transplantation experiments

In the series PTM a block of tissue, containing several myotomes of one side, together with the notochord and the spinal cord of this region, was grafted upon another embryo. An attempt was made to free the side of the spinal cord and the notochord entirely from the adhering cells, whereas the myotomes and the covering skin were disturbed as little as possible. This block of tissue was then transplanted into a deep longitudinal slit in the flank of another embryo. Different experiments were made in which the free surface of the grafted spinal cord was brought into contact with different regions of the host's somites. No tissue of the host was removed, the implantation simply being pressed into the slit and kept there under the pressure of a piece of cover-slip for about thirty minutes. The wound generally healed per primam. In the first days the embryos often were curved, but in the later stages the animals became straight again and showed a normal swimming movement. The zone of the graft was characterized by a dorsal fin developed by the implanted piece on the flank of the host (fig. 2). The area of the implantation proved to have its own innervation. In the case of tactile stimulation of the implanted myotomes, these reacted by lively twitching, whereas stimulation of the host's areas farther away resulted in a normal swimming reaction of the host. These facts will be described more fully in another paper.

LACK OF SPINAL GANGLIA CAUSED BY COMPLETE REMOVAL OF THE SOMITES

In this section the main question is the development of the spinal ganglia in regions where the somites are entirely ex-

cised. This may be answered by the extensive description of a few cases and by a table giving the complete data, obtained in all the experiments.

Case PES 7. The embryo was operated on just after closing of the medullary folds. The mesoderm of the right side along the level of the pronephros was excised, the plane of the cut along the medullary tube being slightly inclined toward the median axis, in order to reach the notochord. The wound was covered by skin taken from the posterior ventral region of another embryo. The wound healed perfectly the same day. During the fifth day after the operation the first contractions appeared. On the following day circulation was observed in the gills. The main zone of defect, indicated by the lack of melanophores, seemed to be in the first segments of the body. During the seventh day, the limb bud of the left side began to grow out, while on the right side it was entirely lacking. The swimming movement was very irregular. On the ninth day after operation the embryo showed an oedema in the ventral region and was preserved.

Cross-sections show that the first and fourth myotomes are partly, the second and third almost entirely, lacking. The spinal ganglion 2 (the spinal ganglia are designated by the number of the somites to which they belong) is present on the normal side in six sections, filling the space between notochord, spinal cord, and somite. The motor root can be traced out from the spinal cord toward the ganglion.

On the operated side there is on this level almost no muscle tissue. There are many cells attached to the spinal cord and the notochord, the origin and definite significance of which cannot be determined. They are separated from the body cavity by a dorsal extension of the splanchnopleure. There are some motor fibers leaving the spinal cord irregularly at this level. In the mass of cells there occur also some scattered cells with nerve-like processes, presumably ganglion cells, but their arrangement is so indefinite that one can hardly call them a spinal ganglion (fig. 3).

Spinal ganglion 3 and a distinct motor root are well developed on the normal side, extending through over five sections. On the operated side again some elements similar to ganglion cells are found, but they lack a compact arrangement and the typical location in the space between spinal cord and notochord.

In the fourth operated segment the somite is partially developed. Here the first typical spinal ganglion appears on the operated side.

From the study of this and several other embryos killed at an early stage, it can be concluded that in the region where the somites are completely removed, no typical spinal ganglia can be found. There are, however, some of the elements (motor fibers and ganglion cells) present, but in a very atypical arrangement.

Case PES 29. An attempt was made to remove the somites from the first to the sixth segment. In the anterior part of the defect region the spinal cord was injured. The wound was covered by ventral skin. Four days later, the first contractions appeared, but on the operated side only head and tail were lifted, following tactile stimulation. Eight days after operation, the animal was swimming rapidly, the limb bud on the operated side was lacking, and the defective region was almost without melanophores. Eighteen days after operation, the animal was killed. The fore limb of the normal side had two digits. The defective region on the left side was whitish and transparent. Branched melanophores were found only on the border of this region.

The sections show that on the operated side the caudal portion of the medulla and the first two segments of the spinal cord are very abnormal. Only a short part in the middle of this region, extending over six sections, is of a normal structure and is covered by a few muscle fibers.

In the posterior part of the operated region the spinal cord exhibits a normal structure. On the normal side spinal ganglion 3 extends through nine sections, spinal ganglion 4 through eleven. At the level of these two segments the oper-

ated region is almost free from musculature, a few fibers occurring near the notochord in several sections. The space between notochord, spinal cord, skin, and the limiting splanchnopleure is occupied by a loose tissue, containing many spindle-shaped nuclei, some leucocytes, connective-tissue fibers and nerve fibers. There is no trace of the typical, heavily staining and relatively large ganglion cells, accumulated into large groups, comparable to a spinal ganglion.

Thus this case again shows the coincidence of the entire lack of somites and the absence of spinal ganglia.

Case PES 75 (fig. 4). The segments 3 to 8 were removed. The embryo was preserved eleven days later and sectioned in an exact frontal plane. It shows very clearly the normal arrangement of the spinal ganglia on the normal side and the complete absence of the somites and the spinal ganglia in the defective region.

Case PES 71 (fig. 5). This represents a series of experiments in which the animals were kept alive for forty-seven days. The procedure in operating was the same as described above, except that a limb bud was grafted into the wound. It fused with the host's limb. An abnormally developed limb resulted. The causes of this will not be considered here. When the animals were killed, the normal fore limb had four well-developed digits and in the hind limbs the fifth toe was appearing. The axial cartilaginous skeleton is well developed throughout the body. The contrast between the operated and the normal side in the defective region is very striking. On the normal side there is strict regularity in the sequence of the spinal ganglia and the cartilaginous pieces, while on the operated side the lack of any segmental structure, as far as the somites were removed, is evident. In the region of segments 4, 5, and 6 only one abnormal cartilaginous piece is present; the spinal ganglia are entirely lacking.

In considering these results, we are led to the conclusion that the complete removal of the somites of a definite region of the body results in an entire suppression of the development of the respective spinal ganglia. This is equally obvious

both in the very young stages and in the animals which were fixed forty-seven days after operation. Neither a degeneration of primarily formed ganglia nor a later regeneration of segmental nervous structures could be found. It will be shown below more fully that the lack of the spinal ganglia is not due to the removal of the ganglion crest cells. There must be, therefore, an inhibition in the development of elements already present. For the nervous elements of the spinal ganglia are not able to differentiate normally and to produce a segmental arrangement independently. The same holds for the arrangement of the motor roots. These facts show conclusively that the spinal cord of the young Pleurodeles embryo does not have an inherent segmental arrangement of its elements.

TABLE 1

Cases with one or more spinal ganglia lacking. Series PES

	NUMBERS OF SEGMENTS AT THE SEAT OF OPERATION AND THE CONDITIONS IN EACH								
5	2D	xF	(4S)						
7	2D	3D	4D	(5S)					
16	2D	3D	(4S)						
27	(2S)	xF	4D	(5S)					
29	xF	3D	4D	xF	(6S)				
30		3D	xF	(5S)					
64		(3S)	xF	(5S)					
65		(3.....4xS)		5D	(6S)				
66		xF	4D	5D	(6S)				
67	xF	3D	4D	5F	(6S)				
68		3D	4D	5D	xF	(7S)			
69		(x.....x.....xS)			6D	7D	xF		
70		xF	4D	5D	6D	xDSk			
71			4D	5D	6D	xF	8Sd		
72		x.....xSk		5D	6D	7D	8D	xF	
73	(x.....x)		4D	5D	6D	7D	8D	(9S)	
75			4D	5D	6D	7D	8D	9D	(10S)
78	2D	3D	4D	5D	6D	7D	8D	9D	(10S)
79			xSk	xSk	xSk	7D	8D	x.....xSk	
f5			4D						
f6							xSd		

x, line of ganglion cells present, but no definite ganglion; ( ), normal spinal ganglion; D, somite entirely lacking; F, some muscle fibers present; S, larger part of the somite present; d, disturbed; Sk, skeleton.

This statement raises the question as to what factors are responsible for the differentiation and for the segmental arrangement of the spinal ganglia.

THE DIFFERENTIATION OF THE GANGLION CELLS

a. The effect of the operation

In the extirpation experiments the somites were removed and the spinal cord and notochord were cleaned of all adhering cells. It might be objected that by this method the ganglion-crest cells were also removed. To test this question, several of the following experiments were carried out on the same stages used in the other experiments. An incision was made between the somites and the spinal cord, the spinal cord and the notochord were left in place and cleaned from all adhering cells, as in series PES, but the somites were not removed. The wound was then pressed together and healed well.

Out of six operated animals five were obtained, one of which was infected and showed abnormal histological conditions.

PESm1 was killed sixty-five days after operation. In the operated region there was a considerable deficiency of each somite, but the skeletal structure of the somite was developed. The spinal cord was entirely normal and the spinal ganglia were all present on the operated side.

PESm4 was operated and raised like PESm1. No trace of defect could be found either in the mesoderm or in the spinal cord and the ganglia.

In the cases PESm2 and 3 the spinal cord was intentionally injured. In both cases the animals were killed thirty-eight days after operation. The mesoderm was defective on the operated side, but the spinal cord was entirely normal. This fact may be emphasized, since in these cases the external surface of the spinal cord was injured intentionally to a large extent, but without any influence on the definitive form of the spinal cord. The spinal ganglia were present and of normal size. Some abnormalities in their form and situation are to

be expected, as they are concomitant with abnormalities in the surrounding mesoderm. These facts will be discussed more fully below.

Thus the series PESm indicates that the methods used did not remove the source of the spinal-ganglion cells. Even if the spinal cord was injured, the spinal ganglia developed and differentiated normally.

This statement is corroborated by some observations on grafted medullary tubes. The anterior and the posterior ends of such tubes are mostly very defective, but if they are found to be surrounded by a normal skeleton and normal somites, they will give rise to full-sized spinal ganglia (fig. 6).

From these observations we can conclude that, in the case of incomplete removal of the mesoderm, slight injury of the spinal cord does not prevent the formation of spinal ganglia and that even a very defective spinal cord is able to form normal ganglia.

b. The rôle of the surrounding mesoderm in the differentiation of ganglion cells

The main difficulty in a histological study of abnormally situated spinal-ganglion cells is to determine which cells are unquestionably ganglion cells. During the first stages of development there is, of course, almost no certainty regarding the significance of a single cell, as there is no reliable and clear distinction between the cells of the sclerotome and those of the ganglion crest. Later, when the skeleton has become partly developed, the ganglion cells differ even from the sheath cells. Their plasma stains dark reddish-brown with Congo red and the round nucleus rather violet-brown with haematoxylin. In order to analyze the conditions under which differentiation of ganglion cells occurs, only animals at this stage of histological development were used.

The first point to be studied is the differentiation of ganglion cells in the case of the complete absence of somites and skeleton. In PES 70, sectioned in a frontal plane, the whole region of defect (segments 4 to 6) is free from muscle fibers

and cartilage. A very loose connective tissue fills the space between spinal cord and skin. This region is entirely devoid of ganglion cells. PES 73 shows the same. Over a distance of 990 μ, muscle and skeleton are absent, and no trace of spinal-ganglion cells can be found (see also fig. 4). In PES 79, there occur on 790 μ of the defective zone several groups of three, four, five, or six differentiated ganglion cells. In the defective region of PES 72, where many leucocytes are accumulated, a large group of ganglion cells is present. But the posterior part of this zone is free from ganglion cells as in the other cases.

We see from these facts that differentiation of typical ganglion cells in the case of complete absence of skeleton and somites is sometimes entirely suppressed, while sometimes a few cells may occur. As the sensory area of the skin on the operated side is still present, we may conclude that the requirements of the peripheral areas are not sufficient to stimulate the differentiation of the ganglion-crest cells under any circumstances.

Another group of cases may be described now in which no differentiated spinal-ganglion cells occurred. In several transplantation experiments the implanted medullary tube is in close contact with muscle material, but without any ganglion cells in its neighborhood. This is found in the cases PTM 5, 8, 14, 19, and 21. Case PTM 21 may be described as an example. The block of tissue containing medullary tube, notochord, and several somites was transplanted in the ventral region of another embryo, the cleaned surface of the spinal cord being near the ventral midline. Sections of the anterior end of the graft show that the medullary tube is in contact with muscle on both its sides and that notochord and skeletal elements are absent in this region. The stratification of the surrounding muscle is abnormal: the direction of many fiber bundles is oblique and even transverse instead of being exactly longitudinal. The spinal cord shows a central canal surrounded by a somewhat irregular gray substance. The periphery of the cord is covered by a layer of loose fibers and

small spindle-shaped nuclei. For 360 μ of this region there are no differentiated spinal-ganglion cells. Posterior to this, some cells appear. At the same level the stratification of the muscles is almost normal and the spinal cord faces a typical medial surface of the somite. The ganglion cells, however, remain scarce, till the first pieces of cartilage appear. Similar observations can be made in the remaining cases 5, 8, 14 (fig. 7), and 19. Where the free stump of the implanted spinal cord does not touch a normal median surface of the myotome, and the skeleton is missing at the same level, no spinal ganglion cells can be found. The first spinal ganglion cells are present as soon as the typical median surface of the myotome begins to appear or normal skeletal pieces are developed.

These observations deserve special attention. It seems remarkable that while, on the one hand, in spite of the presence of a well-developed spinal cord and a quantity of muscle fibers, the spinal-ganglion cells fail to differentiate, on the other hand a very defective stump of the spinal cord, surrounded by normal mesodermal structures, gives rise to normal spinal ganglia. The main condition concomitant with the lack of the spinal-ganglion cells, is the lack of a median myotome surface. It seems strange that a normal median surface of the myotome should be necessary for normal differentiation of the ganglion cells, but other facts seem to corroborate this point of view.

The differentiation of spinal-ganglion cells is remarkably decreased and changed when the free surface of a grafted spinal cord is brought into contact with the lateral surface of the somites. This is found in the cases PTM 15, 16, 23, 25, and 27. PTM 15 and 16 are younger stages, killed nine days after operation. The gills are developed, but not yet branched and the yolk in the intestine has not been resorbed. The free surface of the implanted spinal cord is in contact with the lateral surface of the host's myotome for a distance of about 620 μ in PTM 16 (fig. 8). On the side of the grafted tube, which carried with it the somites, five separate ganglia can be

counted. On the operated side only traces of the removed somites are found, so that this side of the grafted spinal cord faces only the lateral surface of the host's myotomes. In the space between notochord, lateral surface of the host's somites and spinal cord almost no crest cells can be found. Instead, a continuous line of crest cells is situated above the dorsal surface of the spinal cord and attached to the lateral edge of the host's somite. Some cells already show the typical round nucleus and dark plasma, characteristic for spinal-ganglion cells. Therefore, there can be little doubt that these cells came from the ganglion crest, but are accumulated in an abnormal situation. Similar observations can be made on case 15. But the somites were not removed as completely, so that there the spinal cord faces the lateral surface of the host's myotomes only for 80 μ. For this short distance the same abnormal position of the ganglion-crest cells can be noted, namely, above the spinal cord on the lateral edge of the host's somite.

The further differentiation of ganglion cells near the lateral surface of the host's somite is brought out by some older stages, which are all sectioned in frontal planes. PTM 25 and 27 were killed twenty-nine days after operation. The skeleton was not yet developed at this time. In 25, five ganglia are present on the undisturbed side of the grafted spinal cord. In the space between the grafted cord and the host's somites no spinal ganglia can be found, but several groups of clearly differentiated ganglion cells occur, in much smaller number, however, compared to the spinal ganglia of the opposite side. The same observation can be made on 27. A few groups of differentiated ganglion cells are present, but not as many by far as on the opposite side. In PTM 23 (fig. 9) the skeleton is developed, also on the operated side of the grafted cord. In this case the contrast between the two sides is more striking than in the foregoing cases. On the undisturbed side there are well-formed and differentiated spinal ganglia, whereas on the operated side only very few differentiated ganglion cells can be found.

We may conclude from the foregoing experiments that the differentiation of numerous ganglion cells only occurs if the spinal cord is in contact with the median surface of the somite. If the spinal cord is in contact with other parts of the somite, little or no differentiation goes on. The cases PTM 15 and 16 show that the cells of the ganglion crest are formed, but that they accumulate in an abnormal region. As there are very few ganglion cells present in later stages, it must be assumed that these cells do not undergo further differentiation, but remain indifferent and form later, at least in some cases, a part of the numerous spindle-shaped cells which frequently surround the spinal cord under such circumstances. Another interesting point is that the space formed by the implanted spinal cord, the notochord, and the lateral surface of the host's somites is not occupied by the crest cells. The cells rather appear to have been inhibited from migrating down into it. This seems to indicate that this space does not represent a region of least resistance from the mechanical viewpoint, but that it must have under normal conditions some special attractive qualities, which seem to be changed into repulsive ones, in the case of the replacement of the medial somite surface by the lateral one.

The foregoing section gives an account of the circumstances under which the differentiation of ganglion cells was partly or entirely suppressed. In the following part an attempt is made to summarize the circumstances, differing from the normal ones, under which differentiation of ganglion cells could be found.

The cases in which the somites were only partially removed, as in Detwiler's ('27) experiments, only need to be mentioned briefly. In these regions differentiated ganglion cells generally occurred, no matter whether the skeleton was present or not. This accounts for many cases in which the somites, especially the median portion, were not completely removed and ganglia found to be present. As the relation between the arrangement of the somites and that of the spinal ganglia will be discussed later, a short mention of these facts will be sufficient here.

The presence of the median surface of the somites seems to be concomitant with the differentiation of ganglion cells. But another series of experiments shows that, even in the case of complete absence of muscle, ganglion cells are differentiated, provided that the cartilaginous skeleton is developed on the operated side.

In the defective zone of PES 79 skeleton pieces are developed for a distance of 730 μ, muscles being entirely wanting in this region. In the anterior and in the posterior part of this area ganglion cells are accumulated. The same observation was made in PES 69 and PES 73.

Three somewhat different cases were obtained in the grafting experiments. In PTM 12, killed eight days after operation, the cleaned surface of the grafted spinal cord is closely adjacent to the intestine. No trace of muscle is present on this side. But a continuous strand of ganglion cells is found in the typical place between the spinal cord and the notochord. PTM 18, 19 (fig. 10), and 20 are similar to PTM 12, in so far as the free side of the grafted spinal cord was adjacent to the intestine. The animals were killed, after the cartilage had been formed. Cartilage is also developed on the side without somites. Numerous well-differentiated ganglion cells were found, this number being higher than in the cases PES, described above. The difference in quantity may be ascribed to the difference in the environment in early stages. In PES the spinal cord faces only connective tissue, and under these conditions very few ganglion cells develop, whereas in PTM the spinal cord faces connective tissue plus intestine, and here, as it can be seen from PTM 12, many ganglion cells are developed. After differentiation of the cartilage these few cells in PES may differentiate into fewer ganglion cells than the larger number in PTM.

In summarizing the circumstances under which differentiation of ganglion cells is observed, we may state the following conclusions:

1. No or little differentiation of ganglion cells takes place in spite of a normal spinal cord, in the case of, *a*) complete

lack of skeleton and somites; *b*) a grafted medullary tube is adjacent to other parts of the somite than its median surface.

2. Differentiation of ganglion cells occurs, even in the case of a very defective spinal cord, *a*) when only the median surface of the somite is left; *b*) when only the cartilaginous skeleton is present.

THE ARRANGEMENT OF THE GANGLION CELLS

After having reviewed the circumstances under which differentiation of ganglion cells could be found, we have to compare now the arrangement of the spinal-ganglion cells in an abnormal environment. The first question to be considered is the relation between segmental mesodermal structures and the segmental arrangement of the spinal ganglia.

In the case of a complete lack of the somites, the few differentiated ganglion cells never show in their arrangement any relation to the metamerism of the opposite side.

The main source of data concerning the arrangement of the ganglion cells is the study of the abnormal areas of the mesoderm near the operated regions. For the study of the segmentation of somites only frontal sections can be used, the determination of the segmental boundaries in cross-sections being difficult and uncertain.

In PES 65, the first traces of the axial skeleton are present. As the animal was not feeding very well, the somites are somewhat hypoplastic. On the operated side, ganglion 2 is normal, as is somite 2. Somite 3 has no clear posterior boundary in its dorsal region, while more ventrally it is complete and clearly separated from somite 4, which is present only in the ventral region. Between the dorsal part of somite 3 and the spinal cord a large elongated ganglion occurs. As soon as the posterior boundary of somite 3 appears, the ganglion divides into two parts, one of which occupies the place, typical for a spinal ganglion with relation to its somite, the other part lying posterior to somite 4. Thus, this ganglion consists of three parts—a dorsal common portion and two ventral processes which appear at the same level as the posterior boundary of somite 3.

In PES 71 spinal ganglion 2 is normal, 3 is abnormal (figs. 5 and 14), the next ganglion caudal to the defect is 9. Two ganglion-like formations which are anterior to ganglion 9 will be considered here, one of which corresponds to ganglion 8 and the other to the segments 6 and 7. Somite 9 is the somite adjacent to the defective area. Its anterior border is attached to a large piece of cartilage, its posterior surface bordering somite 10. On a more dorsal level no longitudinal muscle bundles occur anterior to somite 9, more ventrally a small group of parallel muscle fibers is found in the region of the seventh segment. Ganglion 9 is round and attached to the anterior border of somite 9 in its dorsal part, whereas its ventral part becomes elongated in an anterior direction, concomitant with the appearance of the muscle bundle of segment 7. This region, on a more dorsal level being free from ganglion cells, also begins to show a strand of ganglion cells parallel to the muscle bundles. These two ganglion-like formations reach ventrally beyond the spinal cord—a relation often found in similar cases. The main points to be brought out are, first, the anterior elongation of both ganglia, and, secondly, the concomitance in the occurrence of a longitudinal muscle bundle and the dorsoventral extension of the ganglion on the level of the seventh segment.

PESf 10 (fig. 11) is a case which shows most clearly the effect of disturbances in the neighboring mesoderm on the arrangement of the spinal ganglia. The posterior part of somite 3 and the anterior of somite 4 are lacking, therefore forming a gap in the row of somites. This gap is filled by loose connective tissue and a large ganglion, which corresponds to a fused third and fourth spinal ganglion. More ventral, where the somites are less defective and touch each other, the rest of the ganglion gives up its intersegmental position and is clearly attached to somite 3, the fourth segment being without any ganglion. This case is comparable to the case PES 65, described above, where as the boundary appears, one part of the ganglion takes its typical place in relation to the somite. The complete somites 5 and 6 possess

only one ganglion, which, however, belongs in its larger part to the posterior border of somite 5. The anterior part of somite 5 is occupied by two abnormal pieces of cartilage. This, the abnormal stratification of muscle fibers and the irregular boundaries of the two somites are the main features of the abnormal mesoderm. A very similar condition is found in PESf 5, where ganglion 5 is lacking. This was accompanied by abnormal development of the skeleton and muscle fibers.

It follows from these and other observations that the segmental boundaries and the stratification of the muscle fibers as well as the skeleton-forming tissue are easily affected by operation. Whenever such irregularities of the mesoderm occur, corresponding disturbances of the spinal ganglia can be found.

In PTM 9 (fig. 12) the host's somites were somewhat affected in their arrangement by the implanted somites of the graft. Especially the boundaries in several segments were very indistinct. Somite 2 is normal, and also its ganglion. The boundaries $^2/_3$ to $^8/_9$ are irregular and sometimes almost missing, some fibers being oblique and ending free. The arrangement of the ganglia in this region is very characteristic. Instead of forming a compact mass in the anterior part of the segment, the ganglion cells extend over the whole segment and are even connected with the cells of the adjacent segment in ganglia 5 and 6. The posterior somites beginning with segment 10 show clear boundaries, and in this region also the normal arrangement of the spinal ganglia is present. The same observations hold in the case of PTM 27.

Since at the time of operation the only segmental structure is the somite, it seems likely that its arrangement was disturbed irreparably by the operation and involved all the other abnormalities of the segmental structures, skeleton as well as spinal ganglia. If this assumption, that the somite is the main factor in segmental arrangement, is true, it should be expected that a shifting of somites of one side of the body must produce a shifting of all other structures by exactly

the same amount. There is some evidence that this assumption is correct.

In the case PTM 23 (fig. 13), sectioned in an exact frontal plane, the grafted medullary tube had brought about a shifting of the host's somites, in comparison with the opposite side. The relations of somite boundaries, skeletal pieces, and spinal ganglia are all typical. The anterior segments are on the same level in regard to the longitudinal axis, but near the graft the segments of the operated side are somewhat drawn back in a posterior direction. Thus results an alternative arrangement of the homologous structures of the two sides in regard to the longitudinal axis. The same shifting of skeletal elements is found in the region adjacent to the defective zone of PES 79. In earlier stages it is present in the cases PTM 27 and PES 75. Here only the somites are shifted, the skeleton being not yet developed. The ganglia are situated according to the boundaries of the somites.

In reviewing the facts given above, the outstanding point seems to be the boundary of the somite and the concomitance of its disturbances with abnormalities in the arrangement of the spinal ganglia.

First, regions with partial destruction of the somite boundary show an abnormal arrangement of the spinal ganglia, so that, instead of being compact, the ganglia are loose and elongated toward the disturbed boundary.

Secondly, ganglia near the free border of a somite exhibit the tendency to elongate beyond the muscle toward the muscleless zone.

In cases 1 and 2, an elongated ganglion becomes rounded and attached to its somite at the level at which the free end of the somite, facing the muscle-free zone, comes into contact with a foregoing somite or the former indistinct boundary becomes distinct.

Thirdly, a shifting of somites in regard to the longitudinal axis brings about a shifting of the spinal ganglia by exactly the same amount.

Fourthly, in the case of mere disturbance of the somite boundaries and without an appreciable loss in mass, in which case differentiation of ganglion cells takes place, the number of spinal ganglia is reduced.

The same arrangement of spinal-ganglion cells can be seen from cross-sections. As example out of many similar cases, PES 69 may be selected. This animal had a limb bud grafted into the posterior part of the defective region. On the operated side the disturbances and the defect in the mesoderm begin to appear at the level of the third spinal ganglion. The skeleton is lacking for 830 μ and parts of the somites are present in the defective area. A strand of ganglion cells, always closely attached to the surface of the somites, extends continuously over the whole region, probably three spinal ganglia, according to the condition of the opposite side, which contains in this distance the spinal ganglia 3, 4, and 5. The anterior end of the ganglionic strand is corresponding presumably to the ganglion 3. A large spinal nerve runs to the fore limb, and a clear dorsal ramus is present. But the dorsal root runs back and enters the spinal cord 320 μ distant from the outgoing motor root for nerve 3, and at the same level as a second motor root. This motor ramus runs for some distance parallel with the ganglionic strand, till a third motor root is entering the strand. At this level a large nerve leaves the strand passing through a gap of the dorsal part of the somite to its lateral surface and running back to the implanted limb on the level of segment 8. The posterior end of the strand again receives a motor root and sends a dorsal root to the spinal cord. Only a kind of dorsal ramus is found in this ganglion and no trace of a ventral ramus. Beginning at this level, the mesoderm, skeleton, and somites are normal as well as the following ganglia. This case shows very clearly how much the arrangement of the segmental nervous structures is affected by abnormalities in the mesoderm, presumably also in the formation of the boundaries of the somites. In a region corresponding to three segments, four motor roots are present, two dorsal roots, two spinal nerves,

and two dorsal rami, together with a continuous strand of ganglion cells. This formation of unsegmented strands of ganglion cells is very frequent in zones where the mesoderm is defective.

The main conclusion to be drawn from these facts is that the segmental arrangement of the spinal ganglia occurs only when there is a degree of normal arrangement of the mesodermal structures, and that in almost all cases disturbances in the mesoderm are accompanied by the abnormal development of ganglion cells.

THE ARRANGEMENT OF NERVE FIBERS

It might be expected that the arrangement of the nerve fibers, outgrowing from the spinal cord, i.e., ventral fibers, would not be affected as much in their segmental arrangement as the spinal ganglia. But that is not the case. In PES 69, as mentioned above, a portion of the spinal cord of the morphological value of three somites produces in this region four separate ventral roots. The same is true in the case of shifting of the somites. The fibers leave the cord exactly according to the boundaries of the somites. Thus, homologous fiber bundles do not leave the cord at the same level on the two sides. These observations indicate clearly that also the location of the outgrowing fiber bundle is changing according to the changes of the surrounding mesoderm and that there is no trace of an inherent metamerism in the nervous system, at least in the stage of operation.

In the regions where skeleton and somites are removed completely, bundles of nerve fibers are found, leaving the cord at irregular intervals and well provided with sheath cells. In many cases these bundles split off in the surrounding connective tissue after a short distance. In case PES 29 such a bundle was found to run through the mesentery to the intestine. Thus the formation of motor fibers never seems to be entirely suppressed in the case of complete removal of the surrounding mesoderm.

The ingrowth of fibers into limb buds also does not seem to be inhibited by the removal of the somites. There is no difference between implanted and original limb buds. In the cases PES 27, 64, 71, and 79 motor nerves were observed running to the limb which were without connection with ganglion cells. Some other peculiarities were found which might find mention. In PES 71 (fig. 14) three large and distinct ventral nerves leave the spinal cord within an area, corresponding to one normal segment, exactly on the level of the limb. This shows clearly how the formation of the motor nerves is without relation to segmentation in the case of lacking somites. A very strange case of outgrowth of fibers was observed in PES 72 (fig. 16). The enormously swollen notochord covers the lateral wall of the spinal cord. In the region of the implanted limb bud, the upper edge of the scapula is very near the medullary tube. In this region on two different levels a bundle of fibers is leaving the dorsal region of the cord. No spinal ganglion cells can be found in the environment. Furthermore, the cells of the dorsal column show the same pigmentation and large size as the ventral nuclei of the opposite side, whereas the dorsal nuclei of the normal and the ventral nuclei of the operated side are comparatively smaller and lying together more densely. An explanation of this case seems difficult at present, but it may range in the same class as the case of abnormal growth of nerve cells described by Nicholas ('24) and found in the case of a limb bud implanted in the dorsal midline.

In general, we see that outgrowth of motor fibers occurs even in the absence of the somites. Especially the nerve supply of limbs is always present.

In the experiments PES we find mostly in the defective region a well-developed external limiting membrane and rarely abnormal and diffuse outgrowth of fibers which occurs, however, in several cases and the circumstances of this behavior of the nerve fibers deserve special attention. Diffuse outgrowth of fibers and an indistinct external limiting membrane is found in an animal (PES 16), the intestine of

which is still filled with yolk. In the same region in which there is the abnormal outgrowth of fibers, the yolk is not resorbed in the notochord and some muscle fibers are disintegrating. The regions lying rostrally and caudally are normal, the notochord is almost free of yolk, and the spinal cord has a clear boundary. It does not seem unlikely that the abnormal growth of the tissues may be due to a kind of infection or sterile disintegration. The behavior of older stages corroborates such an idea (fig. 15). The tissue of the notochord shows an abundant growth, formation of thick masses of fibers, which stain differently from normal notochord tissue. In these regions numerous leucocytes with different forms of nuclei are found. Concomitant with these changes in the environment, the spinal cord sends out, especially on its ventral surface, many irregular fiber bundles which stain very dark with Congo red and contain many spindle-shaped nuclei. In this part of the spinal cord there is no trace of an external limiting membrane. The same observation could be made on the behavior of grafted medullary tubes. In regions which showed great numbers of leucocytes and abnormal growth of connective tissue, diffuse outgrowth of dark-stained nerve fibers could be noted.

In grafted medullary tubes there is also a defective membrana limitans externa and an irregular outgrowth of fibers, if the surrounding mesoderm is very deficient, i.e., especially if the skeleton fails to develop. But parts, even of a very abnormally growing tissue, show a kind of membrane. The membrane also in these cases is the most indistinct, if there occurs abnormal growth of the surrounding tissue and accumulation of leucocytes. If, on the other hand, a very defective tube is surrounded by a perfect vertebral canal, a clear limiting membrane is found.

These observations lead to the conclusion that an extensive lesion of the spinal cord in early stages does not cause the absence of the external limiting membrane, and that even a very defective cord, situated in a normal mesodermal environment, shows a clear external limiting membrane. On the

other hand, diffuse outgrowth of fibers and formation of many spindle-shaped nuclei is always found if the surrounding tissue shows signs of alteration, with leucocytes and abnormal growth of the notochord. In the case of Pleurodeles, it is not possible to decide whether this alteration is due to sterile disintegration of tissue or bacterial infection.

DISCUSSION AND SUMMARY

The foregoing results on the differentiation of ganglion cells, the segmental arrangement of the spinal ganglia, and the outgrowth of nerve fibers are the main points to be discussed here.

In considering the factors involved in the differentiation and the arrangement of the ganglion cells, it seems necessary to call the attention to the importance of distinguishing clearly between histological differentiation and the typical arrangement of histologically differentiated material. Especially in the study of the earliest development of the medullary plate, the stress was laid only on the differentiation of the typical cylindric epithelium, while the arrangement of this tissue, being different in different cases, rarely was taken into account. For instance, Goerttler ('26) is able to show the presence of medullary material without underlying mesoderm, but he does not point out clearly that there exist in the arrangement of this tissue great abnormalities which are less striking in the region where mesoderm is present. It can be seen from these experiments, as from many others, that pure differentiation is not necessarily connected with normal arrangement. But it might be expected that a minimum in the normal arrangement has to be reached in order to build up the stages of development which follow. Therefore, the factors causing the normal arrangement of histologically determined tissues deserve the same attention as has been given to the factors determining histological differentiation.

It was found that differentiated ganglion cells are absent or very scarce in the case of complete lack of skeleton and

somites. As far as it could be determined, in young stages the ganglion-crest cells are migrating ventrally in the defective as in the normal region. But instead of forming aggregates, they remain scattered along the spinal cord, transform into spindle-shaped cells, and show in many cases fibrous processes. These cells seem to lack a stimulus which causes them to transform into ganglion cells. The observations show that this stimulus is acting in the case of adjacent medial surface of the somite or in the presence of cartilage. The lateral surface of the somite lacks this stimulus and exerts a very different influence on the position of the ganglion-crest cells. We are led, therefore, to the conclusion that the muscle, as such, does not exert this stimulus, but that only its medial surface has this quality. An equivalent stimulus seems to be exerted by the cartilage of the axial skeleton. Concerning other forms, our knowledge about the conditions under which spinal-ganglion cells differentiate is not very complete. Harrison's paper ('10) gives the only data on differentiation of ganglion cells in altered surroundings in amphibians. In one case, where a piece of spinal cord was transplanted to the belly, he found a ganglion containing some young ganglion cells. Some data are also obtained on this subject in the chick by chorio-allantoic grafts. Differentiation of ganglion cells generally was found by Danchakoff ('24) and Hoadley ('25). From this there is no clear information as to how the ganglion-crest cells develop in absence of muscle and cartilage. It is evident from one of Hoadley's cases that crest cells can differentiate independently from the spinal cord. The most exact and clearest data are given by Detwiler ('26 b, '27) on the controlling influence of the sensory area on the number of differentiated ganglion cells. But these functional stimuli are only able to work if the ganglion cells are accumulated in more or less normal ganglia. Otherwise, if the morphogenetic stimuli are lacking, as in the case of complete absence of somites, even the presence of skin and its functional requirements cannot stimulate the differentiation of ganglion cells. Therefore, the morphogenetic stimuli

seem to be the initiating, and the functional stimuli, the secondary and controlling factors in the development of spinal ganglia.

That the arrangement of the differentiated ganglion cells is not necessarily segmental was first shown by Shorey ('09) in the chick embryo, and this is also seen from the present experiments. The tendency of the ganglion cells to form long strands or sometimes irregular aggregations is obvious in all cases where the mesodermal environment is abnormal. The observation of Jenkinson ('15) shows something similar in the frog embryo. If the somites are fused in the median plane and the spinal cord has an abnormal basal mass, as is typical in these cases, the spinal ganglia are fused in the median plane. The main factors determining the arrangement of the spinal ganglia seem to be localized in the surroundings of the spinal cord. The ganglion cells themselves show very little inherent tendency for a definite and regular arrangement, except to accumulate in strands. Our results indicate the concomitance of a typical arrangement of spinal ganglia and a distinct boundary of the somite. In regions where the boundaries of the somites are indistinct, the normally compact ganglia break up into strands of cells, and a shifting of the ganglia is always accompanied by a shifting of the somite boundaries.

In regard to the behavior of the spinal-ganglion cells the somite seems to have very definite properties. Its median surface stimulates, its lateral surface inhibits differentiation of ganglion cells, and its boundaries determine the segmental arrangement of the spinal ganglia. Therefore, some differential must be present in the somite in the mediolateral direction, which accounts for the different behavior of the ganglion cells on the two surfaces. And another differential must be present in each somite in anteroposterior direction, maintained by the intersegmental boundaries. It is, of course, not possible to decide whether these differentials are represented by a definite distribution of specific chemical substances or electrical charges.

Considering the new facts revealed by the foregoing experiments, we may now summarize in brief the factors involved in the early development of the nervous system of amphibians:

Two factors are known which determine histological differentiation of the medullary material in the gastrula. They are at least partially overlapping in their effects. One of them is localized in the medullary substance itself, the other is present in the roof of the archenteron. The arrangement of the differentiating medullary substance is controlled by the underlying mesoderm. The formation of the neural folds and the distribution of the medullary cells are dependent on it. The form of the closed medullary tube is developed according to the behavior of the surrounding somites. At these early stages also the quantity of the formed medullary substance is determined by the mesoderm. After closing of the folds, the spinal cord seems to become independent on the surrounding mesoderm as far as the rate of proliferation is concerned. Various factors within the spinal cord itself affect its further growth. (For further information, see Detwiler, '26 a.) The source of the ganglion-crest cells also seems to be determined at this time (Harrison, '25). Their differentiation and arrangement, however, are determined by the mesoderm and their rate of proliferation by the sensory area. Finally, the segmental arrangement of both motor roots and spinal ganglia is under the influence of the segmentation of the mesoderm.[3] This picture of the earliest development of the nervous system in amphibians indicates in spite of the

[3] The considerations here set forth contrast strongly with the views of Child ('21): "The mesodermal theory of segmentation is based primarily on morphological speculation rather than on physiological experiment and it seems to me to offer little prospect of interpretation or synthesis of the various facts as they now stand, and particularly of the very early participation of the ectoderm and its earliest definitive differentiation the nervous primordium in the repetitive process. The facts apparently indicate that segmentation begins in the ectoderm and that the processes in this the most active and most advanced body layer affect conditions in other layers. The segmental ganglion is then the primary definitive organ of the segment as the cephalic ganglion is of the individual" (Chapter VIII, p. 141).

incompleteness of our present knowledge, a great variety of interacting factors within the spinal cord and in its environment.

The fact that, at least in Pleurodeles, the segmental arrangement of the segmental nervous structures is determined by the somites is interesting from a comparative anatomical viewpoint (Naef, '26). As seen from Coghill's work ('13), the nuclei within the spinal cord are arranged continuously and do not show a trace of metamerism. Segmentation varies in some groups of vertebrates to a considerable extent, without affecting the general morphological structure. Variety in the number of body segments seems more easily conceivable, if we assume, on the basis of the foregoing experiments, that the only segmental nervous structures are formed always with reference to the somites, so that a change in the number of somites would produce automatically all other conforming changes in segmentation.

The observations on outgrowth of fibers need only brief discussion. The observations on the outgrowth of motor fibers in regions without somites are in accordance with Harrison's results, and the attractive influence of limb buds on nerve fibers, which was found in all my cases, fits well into Detwiler's results.

The observations on abnormal outgrowth of fibers seem likely to necessitate modification of Hoadley's and Detwiler's views on the external limiting membrane. Hoadley first, in 1925, thought that the abnormal outgrowth of nerve fibers in some mesencephalon grafts might be due to an extensive injury of the external limiting membrane in earlier stages. Detwiler ('25) adopted this view. There is, however, at least in amphibians, in these stages of operation no typical external limiting membrane present. Furthermore, it is obvious from the writer's experiments that extensive injury to the spinal cord in these stages does not produce abnormal growth of fibers or a lack of the external limiting membrane. It is found that in regions with abnormal connective tissue and many leucocytes the external limiting membrane may be

partially lacking and abnormal outgrowth of fibers may occur. Therefore, it is thought that an abnormal environment rather than injury to the spinal cord may affect the external limiting membrane. The abnormal hyperplasia described by Nicholas ('24) in the case of a limb bud, implanted in the dorsal midline, cannot be explained by assuming an injury of the external limiting membrane. It is much more likely that the entirely changed environment produced this kind of growth. In this case, however, the main factor determining the abnormal hyperplasia seems to be the implanted limb bud, as Nicholas assumed. For it can be seen from the slides which Doctor Nicholas kindly showed me that there is in these regions neither accumulation of leucocytes nor abnormal growth of connective tissue.

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PLATE 1

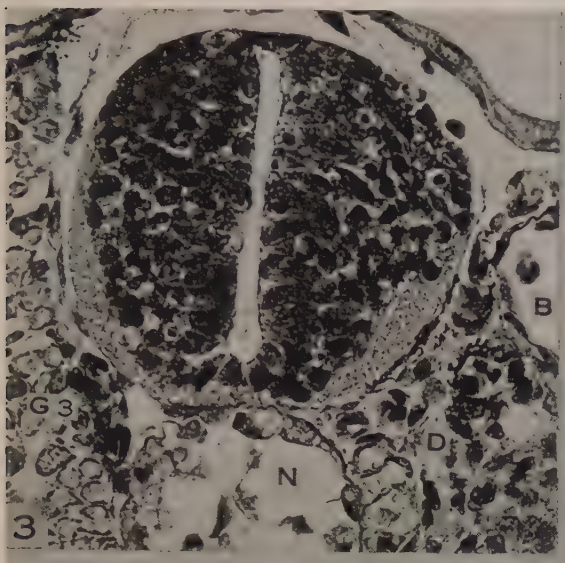
EXPLANATION OF FIGURES

3 (This and all the following figures are microphotographs.) PES 7. Killed nine days after operation. × 250. Cross-section. On the normal side spinal ganglion 3 (*G3*) is developed, whereas on the operated side (*D*) there are only a few cells, and the body cavity (*B*) extends almost to the spinal cord. *N*, notochord.

4 PES 75. Killed eleven days after operation. × 100. Frontal section. The normal side shows the ganglia 3 (*G3*)–7; on the operated side (*D*) the region posterior to ganglion 3 (*G3*) is entirely free from somites and spinal ganglia.

5 PES 71. Killed forty-seven days after operation. × 75. Frontal section. The normal side shows the ganglia 4–6 (*G6*) and the operated side ganglion 3 (*G3*) elongated toward the defective region (*D*). There are no ganglia in the defective region.

6 PTM 14. Killed sixty-five days after operation. × 75. Cross-section through the posterior stump of the grafted spinal cord (*M*), which is surrounded by a complete vertebral canal and normal musculature. A normal spinal ganglion is present (*G*), the corresponding ganglion on the other side is not on this section. *N*, notochord.


B
G 3
D
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3

M
G
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6

G3
G3
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G3
D
G 6
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PLATE 2

EXPLANATION OF FIGURES

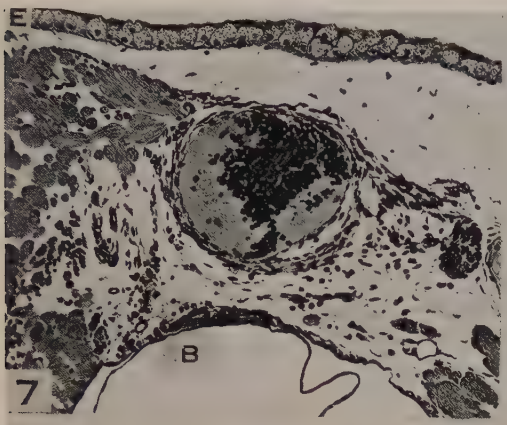
7 PTM 14. Cross-section through the anterior stump of the grafted spinal cord. Neither axial musculature nor skeleton is present. Their is no differentiation of ganglion cells. *E*, epidermis, *B*, body cavity.

8 PTM 16. Killed nine days after operation. × 300. Cross-section of the grafted spinal cord. The lateral surface (*L*) of the host's somite is on the left side of the figure. The crest cells of the operated side (*C*) are situated above the spinal cord. Between the medial surface (*S*) of the grafted somite and the spinal cord there is a normal spinal ganglion (*G*). *N*, notochord.

9 PTM 23. Killed sixty-five days after operation. × 75. Frontal section of the grafted spinal cord. Skeleton, formed by the graft, is present between the lateral surface (*L*) of the host's somites and the spinal cord. On this side there are no ganglion cells. On the side of the grafted somites (*S*) normal spinal ganglia are formed.

10 PTM 19. Killed sixty-five days after operation. × 75. Cross-section. Between the muscleless side of the grafted spinal cord and the host's intestine (*I*) cartilage is developed. On this side spinal-ganglion cells (*G*) are differentiated. Compare figure 7. *S*, somite of the graft; *N*, notochord.

11 PESf 10. Killed sixty-five days after operation. × 25. Frontal section. Note the deficiency of the somites 3 and 4 (*3,4*) of the operated side (*D*), the indistinct boundaries of the following segments and the irregular form of the cartilaginous pieces.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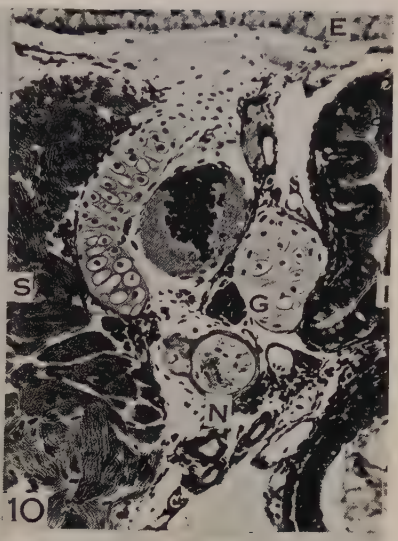
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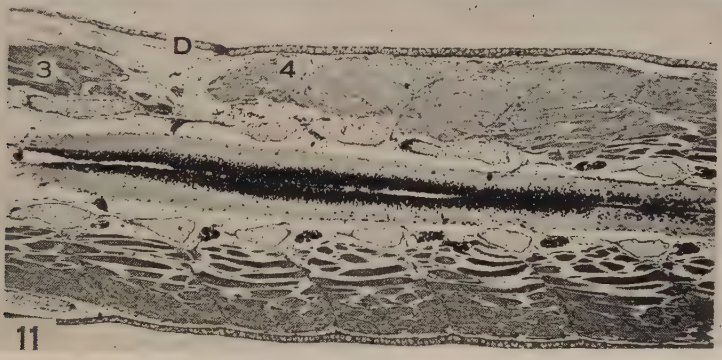
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THE EFFECT OF THE EXTIRPATION OF THE LENS RUDIMENT ON THE DEVELOPMENT OF THE EYE IN AMBLYSTOMA PUNCTATUM, WITH SPECIAL REFERENCE TO THE CHOROID FISSURE

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FIFTY-THREE FIGURES

INTRODUCTION

The eye of amphibians has long been an object for investigation of the problems of self-differentiation and the interaction of parts on each other during development. In the majority of papers treating this subject attention has been directed to such questions as the self-differentiating ability of the lens-forming material, its dependence on the presence of the optic cup, the ability of the optic vesicle to invaginate independently (without the presence of the lens), the ability of the optic cup to differentiate into normal retinal layers and the tapetum, the development of the cornea, et cetera.

Prof. Ross G. Harrison directed my attention to a peculiarity in the choroid fissure in the eye of Amblystoma punctatum occurring in a series of experiments which involved the removal of the lens anlage. There seemed to be complete failure of the choroid fissure to close. In this form a lens does not develop from the lens-forming ectoblast in the absence of the optic cup (LeCron, '06). Neither will a lens develop from strange ectoblast transplanted over the optic cup, although a lens will develop from lens ectoblast transplanted elsewhere.[1] This condition renders Amblystoma

[1] A peculiar condition occurs in Amblystoma punctatum, since a lens will develop from the normal lens-forming area when it is transplanted to another region of the embryo, although it fails to do so, as LeCron ('06) has shown, when it is left in its normal position if the optic cup has been removed (Harrison, '20).

punctatum a very favorable subject for the investigation of the influence of the lens on the differentiation of the optic cup and especially on the behavior of the choroid fissure. It is in particular with the influence of the lens on the behavior of the choroid fissure that this study is concerned.

Through the courtesy of Doctor Harrison, the equipment of the Osborn Zoological Laboratory at Yale was put at my disposal for the investigation of this problem. I wish to thank him for his generosity and friendly cooperation.

A review of the literature on the results of experiments on the amphibian eye is unnecessary, since Werber ('18) has recently summarized them so completely. Relatively few references to a peculiar behavior of the choroid fissure in the absence of the lens appear in the wealth of literature in this field. Such as there are seem to be incidental and the condition recorded to be only in connection with some other problem, while its significance is never discussed.

That the optic cup in amphibia is considered a self-differentiating organ in the absence of the lens is obvious from the conclusions of many workers. For instance, King ('05) says in regard to the eye of Rana palustris: "The eye-forming tissue of the forebrain appears to have the power of self-differentiation as it will develop into a more or less normal cup when removed from its connection with the rest of the brain and without the presence of the lens."

Similar conclusions have been reached by Lewis ('04, '06, '07) for Rana palustris and Rana sylvatica. He finds that in both these forms as early as the late neural-plate stage the optic vesicle possesses "remarkable powers of self-differentiation." This condition has been described in Triton taeniatus by Spemann ('95), in Bombinator by Fischel ('14), and in Salamandra maculosa by Fessler ('20).

Although definitely differentiated so far as the retina, the tapetum, and the optic nerve are concerned, the optic cup is described as variously folded or collapsed through the failure of the chambers of the eye to develop. The smaller chamber and the unexpanded condition of the eye Lewis ('04) ascribes to the absence of vitreous humor.

I find but three references to a peculiar development of the choroid fissure. Lewis ('07) says: "Among the transplanted eyes there are many interesting variations, in size, shape and differentiation. . . . The choroid fissure is present in a number of the transplanted eyes, both regular and irregular ones." Spemann ('12) says of Rana esculenta: "Der fötale Augenspalt ist häufig, namentlich an jungen Augen, unvollkommen ausgebildet, der Teil zwischen dem Rand und dem Grund dieses Spaltes entsprechend zu klein (z.b. Fig. 57). . . . Auch der fötale Augenspalt entwickelt sich weiter; in einem sehr charakteristischen Falle ('07, Fig. 37) nähern sich seine Ränder an der Iris bis fast zur Berührung, um dann weit auseinander zu treten, so dass der Augennerv die längste Strecke seines Verlaufes offen zutage legt." Fessler ('20) noticed in Salamandra maculosa that the ventral half of the cup differed from the dorsal in some cases. He says: "In der ventralen Hälfte fehlt im Gegensatze zur dorsalen die Pars iridica retinae. Das äussere Blatt des Augenbechers geht unmittelbar in den verdickten Abschnitt des innern über, der der Pars optica retinae entspricht. Wie dieser Umschlagsrand so sind auch die Ränder der fetalen Augenspalte beträchtlich verdickt. Im Bereiche dieser massigen Umschlagsränder finden sich Mitosen in grösserer Anzahl als im übrigen Augenbecher vor."

It is evident from these more or less incidental comments that the incomplete development of the choroid fissure in the absence of the lens is not widespread in the forms worked on and that it occurs with no such regularity as I find it in Amblystoma punctatum. I propose, therefore, to give the results of my experiment carried on in this form.

Two lines of experimentation were undertaken; first, by removing the lens anlage and replacing it by non-lens-forming ectoblast to determine the influence of the absence of the lens on the development of the optic cup, especially on the choroid fissure; second, the rotation (180°) of the optic cup both with the lens present and without the lens-forming tissue to determine the specificity of the choroid fissure. Incidentally, also

the problem of the regeneration of a lens from the optic cup arose.

MATERIAL AND METHOD

Amblystoma punctatum was used entirely in the experiments in this paper. The method employed was that described by Harrison ('18). The embryos operated on were in stages from 19 (neural fold closing) to 22 (neural fold closed and fused).[2] In one set of experiments, the most frequent stage used being 21 (fig. 1), the ectoblast over the optic vesicle of the right side was removed to insure the complete elimination of lens-forming material (dotted line). A piece of ectoblast from the branchial region of another embryo of about the same age which had been stained in Nile-blue sulphate (Detwiler, '17) was grafted in its place.[3] In this stage the optic vesicle is a wide-open evagination from the forebrain, just touching the surface ectoblast (fig. 19). Invagination to form the optic cup occurs somewhat later. Only those embryos were kept which showed after recovery from the operation that the optic vesicle was completely covered with foreign ectoblast. In a second series of experiments the whole optic vesicle previous to invagination to form a cup (stages 20–21) was rotated 180°. In some of these the native ectoblast was rotated with the vesicle,[4] in others a graft of foreign (usually branchial) ectoblast replaced the native ectoblast. Embryos were killed at various stages of development in sublimate acetic and stained in Delafield's haematoxylin and Congo red.

[2] The stages referred to by number are those of normal plates of Amblystoma punctatum prepared under the direction of Doctor Harrison, but not yet published.

[3] Staining the graft makes it possible to follow it after it is completely healed in and determine with certainty that the whole optic vesicle is covered with foreign ectoblast.

[4] These grafts were watched and drawn at frequent intervals until they were completely healed in to be certain that they did not rotate back into the original position.

ABBREVIATIONS FOR ALL FIGURES

A., anterior
aboc., anterior border optic cup
atoc., anterior thickening optic cup
ccf., closing choroid fissure
cf., choroid fissure
crl., collapsed retinal layer
D., dorsal
dri., dorsal rim of iris
droc., dorsal rim of optic cup
dtr., dorsal thickening of retina
e., eye
eb., extra balancer from graft
er., everted retina
f., fold of thickened retina
g., graft
ir., iris rim
l., lens
L., lateral
lc., line of cut
Lft., left side of embryo
M., median side of embryo
n., neck
np., nasal pit
oc., optic cup
on., optic nerve
os., optic stalk
ov., optic vesicle
p., pupil
P., posterior
pboc., posterior border optic cup
pc., pupil cavity
pfo., primitive forebrain
proc., posterior rim optic cup
ptoc., posterior thickening optic cup
R., right side of embryo
rfld., retinal fold
rl., retinal layer
rlns., regenerated lens
rp., retinal plug
t., tapetum
sg., surface groove
tdboc., thickened dorsal border optic cup
tr., thickened retina
V., ventral
ves., vesicle of unknown origin
vri., ventral rim of iris
vroc., ventral rim optic cup

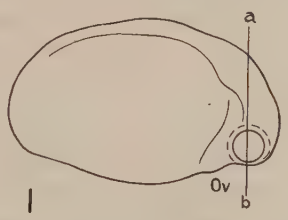


Fig. 1 Camera drawing of an embryo of Amblystoma punctatum, neural fold closed (stage 21) on which most of the operations were made. The line *a-b* indicates the plane of the section shown in figure 19. The dotted circle indicates the extent of the ectoblast removed and replaced by foreign ectoblast. × 10.

EXPERIMENTS

1. Foreign ectoblast (non-lens-forming) substituted for the lens-forming ectoblast

Sections were made and studied of ninety-two embryos in which the lens-forming ectoblast over the right optic vesicle had been removed and replaced by a graft of ectoblast usually

from the branchial region of another embryo, but occasionally from the flank. The result was the same with the two kinds of grafts. These embryos fall into two classes at once: those in which no lens is regenerated (there are sixty-eight of these) and those in which a lens has regenerated or young lens tissue is present (there are twenty-four of these).

A. No lens regenerated from the iris. In the sixty-eight embryos in which no lens is regenerated great abnormalities in the formation of the optic cup appear, especially in regard to the closure of the choroid fissure. In eleven of these, however, even though the eye is variously folded, the choroid

TABLE 1

Showing the classes into which the embryos can be divided as the result of replacing the lens-forming ectoblast by foreign ectoblast

		Total
A. No lens regenerated		
a. Abnormal choroid fissure, i. e. open		57
Group I. Dorsal and anterior edge of optic cup folded	25	
Group II. Base of cup pushed out into pupil	24	
Group III. Optic cup completely everted	8	
b. Choroid fissure normal or nearly so	11	11
B. Lens regenerated from dorsal rim of optic cup, eye normal	24	24
Total embryos studied		92

fissure is normal or nearly so. This group will be described later. There are then fifty-seven without lenses in which the optic cup is variously folded and in which the choroid is abnormal. These, however, can be again sorted into three groups having the same general modifications.

a. Abnormal choroid fissures.

Group I. In this group, of which there are twenty-five representatives, the lips of the choroid fissure seem to be inhibited in their growth so that the fissure remains open, while the dorsal and anterior edge of the optic cup grows to such an extent that it becomes amazingly folded. The result of this is to force the pupil into a posteroventral position.

This effect of the operation is evident very early, as shown in a young stage (fig. 20). The embryo in question was operated on in stage 23, branchial ectoblast from Rana pipiens being grafted over the optic vesicle. It was killed four days later, when it had reached stage 31. In this stage no lens is yet developed in the normal eye. Comparing the right (operated) eye (fig. 20) with the left (normal) eye (fig. 21) it is evident that the optic cup is shorter dorsoventrally than the normal one and slightly more oblique in position. The retinal layer of the optic cup is somewhat thicker, while the tapetal layer and especially the dorsal portion of it which

TABLE 2

Group I. Without lenses and with the dorsal anterior wall of the optic cup thickened, pupil ventroposterior, and with incomplete choroid fissure

a. No folding of the retinal layer		13
Young when killed	6	
Old when killed	7	
b. Excessive growth resulting in folding of the dorsal and anterior wall of the retina	7	7
c. Touching or fusion of dorsal lip with inner surface of cup	5	5
Total		25

forms the dorsal rim of the cup is noticeably thicker than normal. This condition holds in all six embryos sectioned at this young stage.

In a few cases (seven) this condition persists in the older stages, producing a fairly normal retina with no folding of the optic cup. A thickening of the dorsoanterior wall is present in these embryos—a condition which makes the lack of growth in the region of the choroid fissure more obvious. Figure 22 is taken from an embryo in which normally the lens is completely cut off from the ectoblast and contains a cavity with the slight thickening of the posterior wall. The choroid fissure is normally closed in this stage (fig. 23). The embryo from which this section was taken was operated on in stage 19, ectoblast from the flank of another embryo being grafted over the optic vesicle. It was killed in stage 37. The

operated eye (right) gives a very characteristic picture (fig. 22). The choroid fissure is wide open here with mesoblast cells lying between the ventral edge of the cup and the ectoblast. The dorsal edge of the optic cup is thickened and bent ventrally, making the pupil face ventroposteriorly. It is to be noticed that the dorsal lip of the cup and for some distance back of it the tapetal layer is in contact with the ectoblast. A reconstruction of the above eye (fig. 2)[5] shows that the whole anterior region also has grown so that the pupil faces ventroposteriorly.

The other six cases agree in general with the conditions described for this embryo. In only one of the six is mesoblast present between the dorsal lip of the cup and the ectoblast—a point which may be of importance.

The posterior position of the pupil is well shown in a frontal section of a still later embryo. This embryo was operated on in stage 19 and preserved at stage 43, at which time the layers of the retina, including the rods and cones, are well developed, the lens lies well within the iris, the lens cavity is nearly closed, and the lens fibers developed (fig. 30). Figure 24 shows a frontal section through the ventral part of the right (operated) eye, including the pupil and optic nerve. The differentiation of the layers of the retina is not different from that of the left (normal) eye except that in the dorsal anterior portion the retina is thicker. The tapetal layer is only slightly thicker than normal at this stage. The edges of the optic cup touch in this region of the eye, so that the posteriorly directed pupil is closed here. A few sections above the one represented in the drawing shows the pupil as a narrow opening. A reconstruction of this eye (fig. 3) shows the pupil as a narrow slit facing posteroventrally and running obliquely in the lateral median direction. This shows very clearly the excess of anterior growth and the failure of the choroid fissure to close. The tapetal layer, because of the excessive anterior growth, is brought directly against the ectoblast, with no mesoblast intervening.

[5] These reconstructions were made to scale from camera drawings of the entire series of sections of the eye.

This dorsal anterior thickening of the optic cup is often carried to such an extent that the dorsal wall by its rapid growth folds on itself. This condition is represented by a group of seven embryos, and this is well shown in an embryo operated on in stage 23 and preserved in stage 37. Figures 25 and 26 give two sections through the right (operated) eye of this embryo, figure 25 being anterior to figure 26. It is obvious from figure 25 that the choroid fissure is not closing

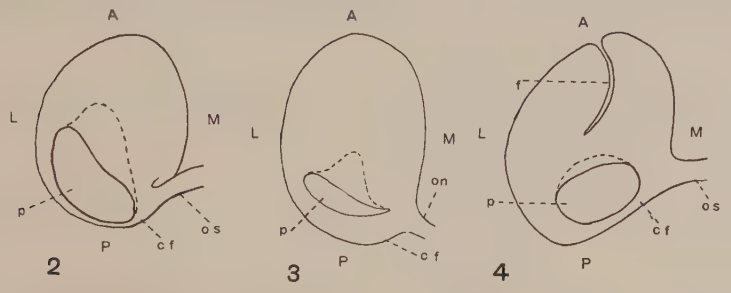


Fig. 2 Ventral view of a reconstruction of the right (operated) eye (stage 37), a cross-section of which is shown in figure 22, showing the failure of the choroid fissure to close, the ventroposterior position of the pupil caused by the thickening and backward growth of the anterior portion of the optic cup. × 55.

Fig. 3 Ventral view of a reconstruction of the right (operated) eye of an older embryo (stage 43), a frontal section of which is shown in figure 24, showing the choroid fissure open, excessive anterior thickening of the optic cup and the ventroposterior position of the pupil. × 55.

Fig. 4 Ventral view of a reconstruction of the right (operated) eye (stage 37), cross-sections of which are shown in figures 25 and 26, in which the anterior thickening is folded and the pupil cavity shallow. The pupil is ventroposterior in position and the anterior border of the optic cup has grown far posteriorly while the choroid fissure has not closed. × 55.

properly, that the opening of the optic cup is directed ventrally, that a mass of retinal cells lies dorsally separated from the rest of the cup by a narrow pigment layer, and that the tapetal layer is continuous around the whole eye cup. That this mass is continuous with the thickened dorsal portion of the retina shown in figure 26 is made evident by tracing the condition found in sections between these two. The fold is obviously directed dorsoventrally. A reconstruction of this eye (fig. 4) shows the fold to extend in the dorsoventral direc-

tion and somewhat obliquely. The pupil is directed posteroventrally and is large, while the pupil cavity is shallow. The lack of growth in the region of the choroid fissure is clear.

Frontal sections through an embryo somewhat older than the last show this excess of anterior dorsal growth and folding still better (fig. 27). From this it is obvious that the folding occurs in the same way as in the previous embryo, i.e., growth occurs in such a way as to make the cleft of the fold run in the dorsoventral direction. The pupil is again posteroventral, as shown in a reconstruction (fig. 5). The lack of growth of the lips of the choroid fissure is especially striking in this eye because of the excessive posterior growth of the anterior rim of the optic cup.

A third type of this excess of dorsal growth is represented by a group of five embryos in which the dorsal edge of the iris has grown so far ventrally that its ventral edge touches and often fuses with the inner surface of the retinal cup. An early stage of this condition is shown in figure 28, which is taken from an embryo that was operated on in stage 21 and preserved at stage 38. The section is toward the posterior end of the eye showing the wide-open choroid fissure with the dorsal thickened border of the retina grown well down toward the ventral surface and turned in toward the cavity of the cup so that it nearly touches the retina. A reconstruction of this eye (fig. 6) is not very different from the previous one, except in the depth of the pupil cavity which extends far anteriorly as a wide slit and in the less thickened anterior portion.

A later stage of this condition is shown in figure 29, which is taken from an embryo operated on in stage 19 and preserved in stage 42. This section is through the posterior portion of the right (operated) eye just in front of the choroid fissure. Here the layers of the retina are differentiated. The contrast between the growth of the dorsal portion of the iris rim and the ventral is marked, the dorsal lip touching and probably fused with the retina. The ventral lip disappears in the choroid fissure a few sections beyond this point. Figure 30 is a section of the left (normal) eye of this embryo.

With all the various modifications of the dorso-anterior wall in this group consisting of twenty-five out of the whole sixty-eight, they all show the same general modifications; the posteroventral position of the pupil, the incomplete choroid fissure, and the tapetal layer against the surface ectoblast.

Group II. In this group without lenses, of which there are twenty-four embryos, the dorsal portion of the optic cup, while somewhat thickened, is not so extreme as in group I, and the pupil is as a consequence more normal in position

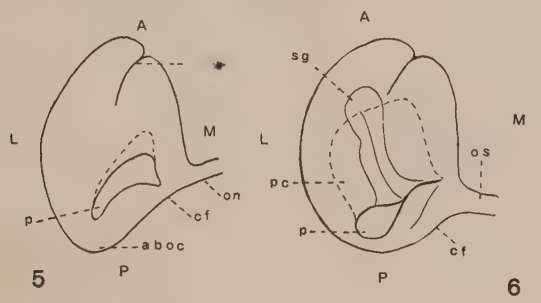
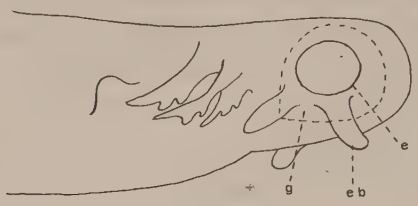


Fig. 5 Ventral view of a reconstruction of the right (operated) eye of a slightly older embryo (stage 39), a frontal section of which is shown in figure 27. Similar to the eye of the previous embryo with a fold in the anterior thickening of the optic cup, but showing still more plainly the excessive posterior growth of the anterior border of the optic cup. × 55.

Fig. 6 Ventral view of a reconstruction of the right (operated) eye, a cross-section of which is shown in figure 28, in which the anterior portion is much less thickened, as shown by the deep pupil cavity, while the posterior growth of the anterior border of the optic cup is extreme. × 55.

and in size, while the middle or base of the cup is pushed outward into the pupil, often filling it. This folding is often associated with a greater growth of the posterior region of the cup. As before, the choroid fissure is wide open. In general, because of the open choroid fissure and of the lessened growth of the dorsal rim of the iris, the pupil, while lateral, is inclined to face more or less ventrally. This lateral position of the pupil is evident in surface view of the living animal. Embryos belonging to group I, in which the pupil is directed ventroposteriorly, naturally give no superficial

evidence of a pupil. Figure 7 is such an embryo drawn in stage 39. It belongs to the group of strongly thickened anterior dorsal wall. The contrast is striking between the condition shown in this group and the condition in group II, as shown in figure 8. Here the incomplete laterally placed pupil, bounded dorsally and laterally but open ventrally, is



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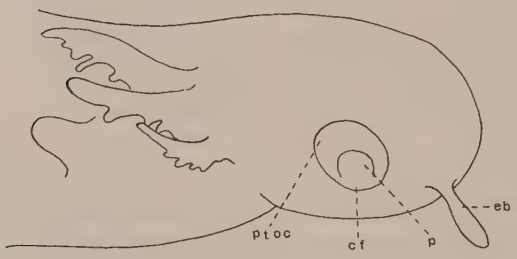

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Fig. 7 Camera drawing of the surface view of an embryo (stage 39) belonging to group I in which no pupil is evident on the surface, since it lies on the posteroventral side of the eye. The dotted line shows the extent of the graft of branchial ectoblast. An extra balancer has developed from this graft. × 17.

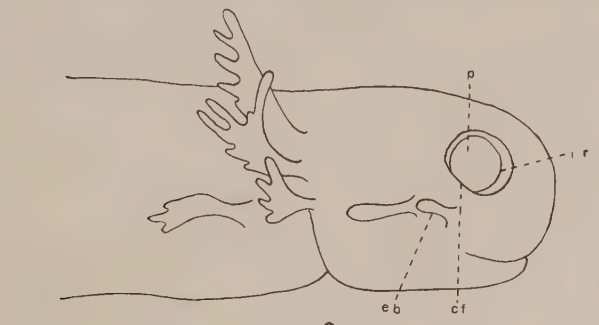
Fig. 8 Camera drawing of the surface view of an embryo (stage 40) belonging to group II, showing the laterally placed pupil, which is incomplete ventrally because of the open choroid fissure, the posterior thickening of the optic cup. A section of this eye is shown in figure 31. × 17.

clear. The posterior thickening of the optic cup which pushes the pupil slightly anteriorly is also evident.

A section of this embryo which is fairly young (stage 40) is shown in figure 31 taken through the region of the choroid fissure, which is wide open, with the optic nerve running along the ventral border of the cup. The retina is somewhat thickened, folded, and bulges into the pupil, while the pupil itself is larger than normal and directed ventrolaterally, the dorsal

tapetal layer only touching the ectoblast. The contrast between the dorsal border of the iris here and in the preceding group is striking.

As the embryo grows older this bulging of the retina apparently increases until usually it is pressed against the surface ectoblast so that it often rolls over the edge of the pupil. Figure 9 shows the surface view of such an embryo operated on at stage 22 and killed at stage 45. The pupil is nearly as large as the optic cup and is incomplete ventroposteriorly.



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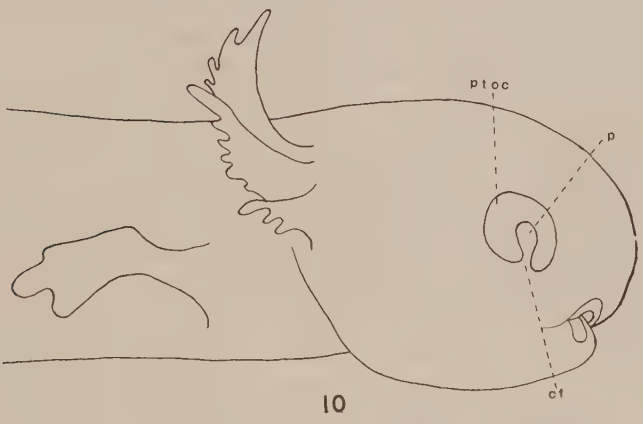

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Fig. 9 Camera drawing of an older embryo (stage 45) belonging to group II, showing the large incomplete pupil. The pupil is filled in this case with a retinal plug shown in figure 32, which has forced the iris rim to the edge of the eye. Figure 33 is a section of this eye through the choroid fissure. × 17.

Fig. 10 Camera drawing of the surface view of a still later embryo (stage 46), showing the open choroid fissure and the posterior thickening of the eye. Figure 34 gives a section through the anterior third of this eye. × 17.

A section through the anterior third of this eye (fig. 32) gives a very characteristic picture of this stage. The whole retina, which is here differentiated into layers, is everted so to speak; i.e., it arches out so that it presses against the surface ectoblast. The rim of the pupil is withdrawn and deeply placed. The tapetal layer is greatly thickened at the back of the eye, while it is quite normal just back of the rim of the iris. Figure 33 shows a section through a region just back of the middle of the same eye and through the wide-open choroid fissure. Here the ventral lip of the pupil is missing and the optic nerve runs along the ventral surface of the optic cup. This eye shows no thickening of the posterior region.

The condition found in a number (fourteen) of late embryos (stages 46 and later) is of interest here in view of another group of embryos of the same age in which the choroid fissure is closed (to be described later). Figure 10 gives a very characteristic picture from surface view of the condition found in these older embryos with the choroid fissure incomplete ventrally, making the pupil open here. The posterior region of the optic cup is thickened, as it is so often in these embryos in which the center bulges into the pupil. A section (fig. 34) which is through this eye in front of the choroid fissure gives an even more characteristic picture of this group than the one just described, for here the retina, while folded and arched out, filling the pupil, is not everted so strongly, there being little space between the retina and the tapetal layer. Sections through the middle of the eye show the choroid fissure wide open.

Group III. In a small group of embryos (eight) the optic cup is very much bulged, often much folded and fused, yet the choroid fissure is open. They are really an extreme case of group II. This everted condition of the eye seems to be associated with a large graft; i.e., the grafted piece being larger than the piece removed, and as a consequence little tension is present in the region of the eye.

This condition is evident in a fairly early stage, as shown in figure 35, taken from an embryo operated on in stage 20 and killed at stage 39. Here the retina is bulged and folded in such a way that a cavity connected with the split that usually occurs between the retina and the tapetal layer is

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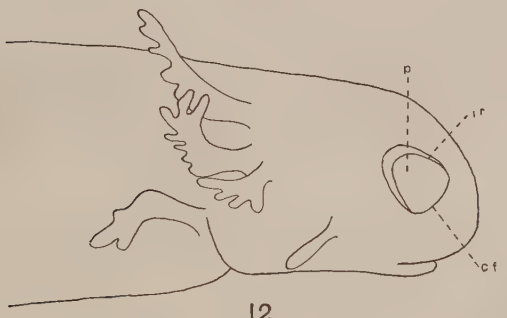

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Fig. 11 Camera drawing of the surface view of an embryo (stage 39), in which the optic cup is everted so strongly that no pupil or iris rim is formed. Figure 35 shows a section through the choroid fissure of this eye. × 17.

Fig. 12 Camera drawing of the surface view of an older embryo (stage 45), in which the retinal layer is strongly everted, but with a small iris rim and with the choroid fissure open ventrally. Figure 36 shows the internal structure of this eye through the choroid fissure. × 17.

carried well toward the surface of the vesicle which is arched against the ectoblast. Pigment like that of the tapetal layer is present in this cavity. Because of this extreme bulging there is no iris rim, the tapetal layer merging directly into the retinal layer some distance back from the surface of the head. This accounts for the fact that in the surface drawing (fig. 11) made before sectioning no pupil was visible.

In a much older embryo this evaginated condition of the eye is so extreme that it was evident in surface view (fig. 12). Here, in contrast to the last embryo, a narrow iris rim is present dorsally and laterally, but absent anteroventrally forming a very large pupil. This embryo was operated on in stage 21 and killed in stage 45. Sections of this embryo show the whole optic cup everted with the thin retinal layer pressed against the surface ectoblast. The tapetal layer is thicker than in the previous embryo and present dorsally as far as the iris rim. The section drawn in figure 36 is through the choroid fissure, showing the optic nerve running along the ventral wall of this everted cup. The choroid fissure is naturally incomplete. This abnormal group is of interest chiefly because of the fact that the choroid fissure has failed to close in every one of the embryos.

In these three groups, which includes fifty-seven embryos without lenses, we find it universally true that the choroid fissure is abnormal; i.e., it has failed to close in the absence of the lens, regardless of the various ways in which the optic cup is folded, fused, or bulged and regardless of the age of the embryos.

b. Choroid fissure complete and no lens present. In a few cases (eleven) in late stages only it was found that the choroid fissure was closed and the optic nerve entered the eye cup normally or nearly so. This seems to be a late adjustment, for no indication of it was found in stages earlier than 43 or 44. In some cases the closure could not have been completed long, since the nerve enters the cup nearer the ventral surface than is normal for the stage (fig. 37).

Six of these embryos belong to the group described above in which the middle of the optic cup is more or less everted into the pupil of the eye. They differ, however, in the position of the eyeball and pupil. In these the eyeball is tipped slightly upward, more nearly like the normal eye, making the pupil lateral instead of ventrolateral, and in all cases studied the edges of the cup come very near to the ectoblast throughout (fig. 13). Only a small amount of mesoblast lies between

the edges of the cup and the ectoblast, i.e., that which is forming the cornea and is present in the normal eye at this stage. A section of such an eye is shown in figure 37, which is taken from an embryo operated on in stage 21 and killed in stage 44. While the choroid fissure is closed, the optic nerve enters the eye more ventrally than on the normal side of the embryo indicating a recent closure. The pupil is nearly lateral

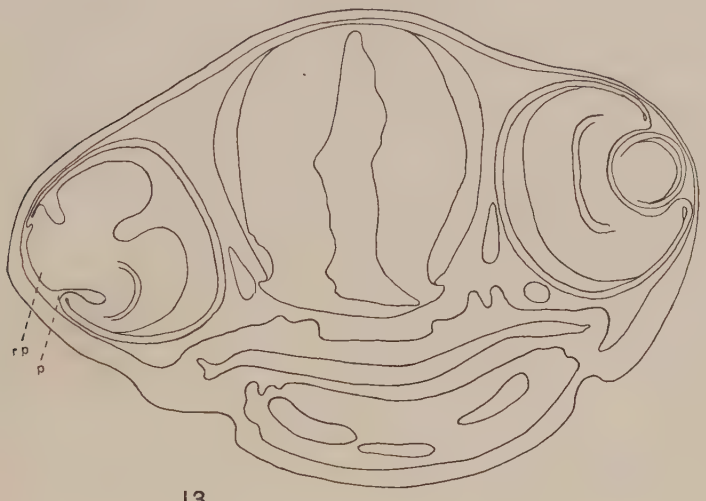

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Fig. 13 Camera drawing of a cross-section of the normal and operated eye in which the choroid fissure has closed in the absence of a lens (stage 44). The right (operated) eye shows that the position of the pupil is nearly normal. Figure 37 is a section through the recently closed choroid fissure. × 55.

in position and is filled with the folded retina. The dorsal lip of the iris is very near the ectoblast as is the whole everted optic cup.

In several cases, in contrast with those eyes in which the retina bulges, the optic cup has collapsed in such a way that the inner walls of the retina have fused and the vitreous cavity is nearly obliterated. Figure 38 shows such a section from an older embryo than the last, probably stage 47 operated on at stage 21. Here again the small pupil is nearly

normal in position (fig. 14), and the optic nerve enters the eye normally, while corneal mesoblast only seems to be present.

It is difficult to understand why these few embryos have been able to complete the closure of the choroid fissure in the absence of the lens, while the overwhelming majority have not. It may be that, under the conditions of the operation,

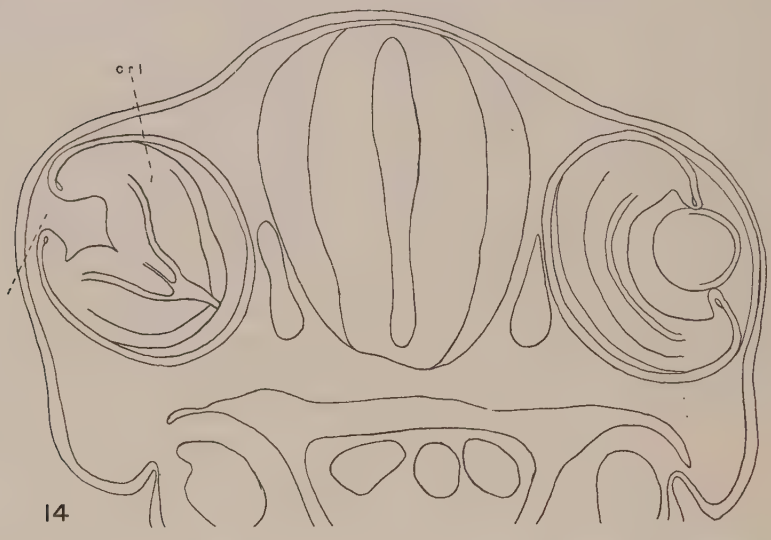


Fig. 14 Camera drawing of a cross-section of a normal and an operated eye in which the choroid fissure has closed in the absence of a lens. The right (operated) eye shows that the position of the pupil is nearly normal, while the optic cup has collapsed. Figure 38 is through this eye in the region of the optic nerve. × 55.

the edges of the cup touched the surface ectoblast throughout their extent, and this condition with the lack of mesoblast between the optic cup and the ectoblast brought about a tardy closure of the fissure. So far as can be seen, no premonition of this condition is present in embryos younger than stage 44.

B. With lenses regenerated from the iris. Twenty-four of the ninety-two embryos sectioned showed either completely regenerated lenses or young lenses in process of development. Only those cases are considered in which it is certain that

all the lens-forming ectoblast was removed and replaced by the graft from the branchial region. In fourteen of the eyes the retina is normal looking and shows no foldings or abnormalities of any kind. In ten the retina is somewhat folded, but in no case is it as extreme as in some of the eyes without lenses. When folding occurs it is of the type of group II, i.e., a bulging or arching of the middle of the cup. I found no lenses regenerated in those eyes in which the dorsal portion of the optic cup shows excessive growth or in those in which the anterior rim of the iris had grown far posteriorly. In all these twenty-four cases the choroid fissure is either closed or in the younger embryos is closing in a perfectly normal fashion.

The origin of the lens is of considerable interest. In fourteen cases where the lens is young and not fully formed it is very obviously regenerated from the dorsal rim of the optic cup. In the remaining ten cases the lens is fully formed and free. It is small and lies against the dorsal rim of the cup and does not fill the pupil opening as it does normally.

The youngest lens tissue which could be determined with certainty was found in embryos of stages 38 and 39. Figure 15 shows an embryo in which a vague clear area toward the dorsal portion of the eye was visible in the living embryo. No outline of the pupil could be located in this young stage. On sectioning it was found that the dorsal border of the iris rim was thickened and clear. Figure 39 shows a section from the anterior portion of this eye, indicating the lens to be anterior as well as dorsal in position—a condition confirmed by the surface view. This condition holds in most of the embryos studied. The tapetal layer is considerably thickened as it approaches the rim of the cup, and the pigment is either absorbed or still unformed. In this embryo I found one lymph cell containing pigment in this region. The inner layer of this dorsal swelling in comparison with the ventral lip is greatly thickened and the nuclei which are somewhat elongated are already arranging themselves in a more or less radial position. A small cavity is present between the two

layers. The cells themselves seem clearer than the other retinal cells.

The rest of the eye is perfectly normal, no arching or folding occurring. Figure 40 from the posterior region of the eye, i.e., back of the lens anlage shows that the choroid fissure has begun to close in a perfectly regular fashion in the presence of this very young lens.

In other embryos (stages 39 and 40) in which for some reason folding of the floor of the cup occurred the lens is formed in the same way. Figure 41 is taken from such an eye, in which again a clear area was seen dorsally in the living embryo. This embryo was operated on in stage 22 and killed at stage 40. The lens is a bit older in this case and it will serve well to show the next step in the regeneration of the lens. The swelling which is present on the dorsal rim of the iris is more definitely cut off from the rest of the cup, the cavity between the two layers is larger and contains a cell which is loaded with pigment. The tapetal layer is clear farther back from the rim and is somewhat thicker at the rim. The nuclei of the inner layer show the same radial arrangement as in the earlier embryo, and the whole layer is somewhat thicker. It is connected with the rest of the retinal layer by a narrow neck. The cells again give the impression of clearness. Even though the retina is arched in the middle and the lens tissue young, the choroid fissure is completely closed.

A still later stage in the formation of the lens is shown in an embryo of stage 39, in which again in the living condition a lens-like structure was visible (fig. 16) in the anterior dorsal region of the eye, while the pigment has not yet marked the outline of the pupil. Sections of this eye show the optic cup normal and unfolded. A section through the anterior third of the eye (fig. 42) shows a lens-like structure nearly cut off from the dorsal rim of the optic cup, attached only by a narrow neck. The cavity of the lens has become larger and crescent-shaped due to the pushing up of the cells of the inner layer which have assumed an elongated clear condition

characteristic of the early formation of the lens fibers. The nuclei of these cells are characteristically pale. The important point here is the connection of the outer layer (lens epithelium) with the tapetal layer of the optic cup and the connection of the inner layer (fiber forming) with the retinal

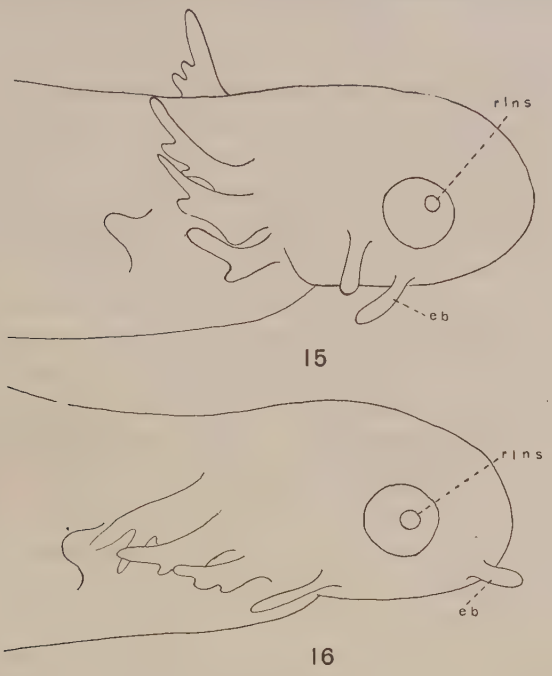


Fig. 15 Camera drawing of the surface view of an embryo (stage 39), in which a young lens regenerating from the dorsal border of the iris was identified in the living embryo as a vague clear area toward the dorsal anterior portion of the eye. Figure 39 shows this lens tissue in section. × 17.

Fig. 16 Camera drawing of the surface view of an embryo (stage 39), in which a definite lens was evident as a clear body in the anterior region of the eye of the living form. Figures 42 and 43 are through this eye. × 17.

layer of the cup. The pigment of the tapetal layer stops at the neck of the enlargement. In this embryo, as in others, the choroid fissure is completely closed (fig. 43).

The completed free lens is found in stages 39, 40, or 41. The lens is small, about half the size normal for this stage, and does not fill the pupil, being pressed against the dorsal

border of the optic cup from which it was presumably cut off. One is struck with the fact that all the small regenerated lenses have this position against the dorsal rim of the iris. Figure 44 is a section showing this condition taken through the region of the choroid fissure which is closed. This embryo was operated on at stage 20 and killed in stage 39. The dorsal edge of the optic cup is still thickened more than is normal for this stage, and the tapetal layer at this point is wider and less pigmented than normally.

Further development seems to be purely a matter of growth and differentiation of the lens fibers. Growth takes place apparently as in the normal lens. An eye which at first glance looks perfectly normal is shown in figure 45, which is taken from an embryo operated on in stage 20 and killed in stage 43. Comparing it, however, with the eye of the left (normal) side, it is evident that the lens is smaller and still pressed against the dorsal rim of the iris, which by this time has become deeply pigmented. The lens also does not quite fill the pupil ventrally as does the normal one. Figure 46 gives an enlarged portion of the dorsal region of the iris, showing the way in which it is still flattened against the lens. The choroid fissure is completely closed and there is no indication of abnormality about the eye.

Of the four cases of older embryos in which a small lens is present in eyes with distorted and folded retinas, two are of interest here.

One case is found in an embryo operated on in stage 22 and killed in stage 41. Sections show a small free lens, which seems perfectly formed, pressed in between the dorsal border of the optic cup and the fold of the retina which is pushed out into the pupil (fig. 47). There is no indication other than its position as to the origin of this lens. It is to be noted that the choroid fissure is completely normal in this eye.

The second case is that of a collapsed eye in which the inner walls of the retinal cup seem fused. This embryo was operated on in stage 21 and killed in stage 46. The interesting thing in regard to this lens is that it lies embedded in

the dorsal edge of the cup from which it has never separated, although well differentiated into lens fibers (fig. 48). Its outer layer is still connected with the tapetal layer. Its inner (dorsomedian) side has no epithelial layer, the lens fibers lying directly against the retinal cells. Here again the choroid fissure is completely normal.

The fact that, even in those eyes in which the lens is only beginning to form, the choroid fissure begins to close and in those forms in which a small lens partly fills the pupil the choroid fissure is completely closed as it is even in strongly folded and abnormal eyes in which a lens is present, indicates a close connection between these two events when compared with the conditions described in the previous section where the lens is absent.

2. Rotation of the optic vesicle

Since the absence of the lens seems to have so definite an effect on the choroid fissure, it seems desirable to determine, if possible, whether the choroid fissure is localized and specific at the early stage in which the above operations were performed or whether it is simply due to its position. This was attempted by rotating the whole optic vesicle 180°. In some cases the overlying lens-forming ectoblast was rotated with it. In other experiments after rotation of the vesicle the lens-forming ectoblast was replaced by ectoblast from the branchial region of another embryo. In the first group, by means of the pigment markings in the ectoblast, the graft was followed until it was well healed in, in order to be sure that there was no rotation or shifting of the position of the vesicle. The operation was performed on young embryos soon after the closure of the neural tube (stages 21 and 22) when the optic vesicle is well marked off from the brain and has just met the surface ectoblast and when the vesicle is still wide open into the neural cavity. Operations were always on the right eye. Thirty such embryos were studied. These embryos were killed between the stages 33 and 40, the choroid fissure being completely closed normally during this period.

In every case the eye from the surface in the living embryo looked perfectly normal.

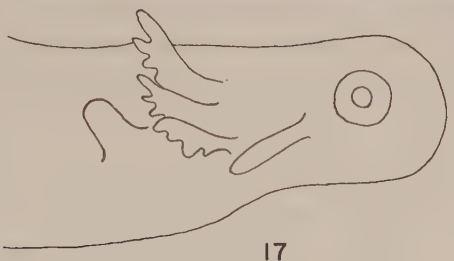
Sections show that even in the youngest stage studied (33) the optic vesicle had united with the brain, and the eye, though somewhat behind the left (normal) eye, was developing in a perfectly regular fashion. The optic cup is formed typically, the lens is a mere thickening of the ectoblast which is typical for this stage. The choroid fissure is being formed on the new ventral side (original dorsal side) of the optic cup. Figures 49 and 50 are drawn from a slightly older embryo (stage 36) in which the lens is more definitely formed. The eye of the right (operated) side has been pushed a little ventrally, while the tapetal layer is somewhat thicker than normal and the pigment has not yet developed in it. The opening of the optic stalk into the neural cavity is wider than on the left side.

That the eye continues to develop normally is shown in a later stage (40), the surface view of which is shown in figure 17. This shows the normal appearance of the eye in the living condition. Sections of this embryo (figs. 51 and 52) show a perfectly normal appearing eye, the choroid fissure closed, the optic nerve running to the ventral surface of the brain as normally as if it had never been cut at this point.

The group of embryos in which foreign (branchial) ectoblast replaced the lens-forming tissue over the optic cup which had been rotated further confirm the conclusion that the choroid fissure is formed ventrally, regardless of what part of the optic cup is present there. Many of these embryos died because of the severity of the operation, and many of those that lived show the eye bulged more or less. Nevertheless, sections through such an embryo (stage 37) as that drawn from the surface, figure 18, show the eye bulging but little. A section of the right eye (fig. 53) is not so very different from those described earlier in which the ectoblast only was replaced. The whole process is somewhat delayed here by the necessary readjustment in forming a new attachment to the brain. That this is done normally is evident from the

figure where the optic stalk is present in its normal ventral position. The retinal layer is thickened and somewhat folded, but the ventral choroid fissure is clear, it having been formed from the original dorsal side of the optic cup.

Since in both sets of experiments the choroid fissure is formed in the ventral position from the original dorsal side



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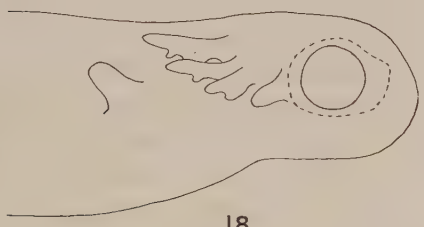

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Fig. 17 Camera drawing of an embryo (stage 39), in which the right optic vesicle and the lens-forming ectoblast were rotated 180° while in stage 21. The eye is completely normal in appearance. Figures 51 and 52 are taken from this eye. × 17.

Fig. 18 Camera drawing of an embryo in which the right optic vesicle in stage 21 was rotated 180°, while the lens-forming ectoblast was replaced by a graft of foreign ectoblast. The eye shows a bulged condition, while no lens or pupil is formed. The dotted line indicates the size of the graft. Figure 53 is through this eye. × 17.

of the vesicle, it seems obvious that the choroid fissure is not specific or localized early, but can be formed from any portion of the optic cup which occupies the ventral position.

DISCUSSION

Choroid fissure. From the foregoing experiments it seems evident that the presence of the lens has a decided influence on the ability of the choroid fissure to develop and close prop-

erly. Whether this influence is chemical or mechanical is not certain from my results. The fact that in those forms where even a small lens was regenerating, the choroid fissure was always closed, even when the cup was folded and distorted, as well as when it was more nearly normal, would suggest a chemical influence. A small regenerating lens not filling the pupil or a very young one still attached to the dorsal border of the optic cup can have very little mechanical influence. However, the fact that, in a small number of late embryos (stages 44 to 47) in which the lens is absent, we find the choroid fissure closed cannot be explained in this way. The large majority of eyes with no lenses present (fifty-seven) show the choroid fissure entirely open ventrally and the optic nerve running along the free ventral surface of the cup. This condition of the choroid fissure is found in embryos of all ages up to stage 47, that is, including late stages in a few cases of which the choroid fissure closed (44–47) so that the ability to close is evidently not dependent on age. It has been pointed out that in the cases in which the choroid fissure is closed the position of the eye is more nearly normal than in those in which it has remained open, i.e., the pupil faces in a slightly dorsolateral direction (figs. 13 and 14), while in most forms in which the choroid fissure remains open the pupil is directed in the ventrolateral direction in varying degrees (figs. 21, 24, 25, 27, 28, etc.). Also the edges of the optic cup lie close to the surface ectoblast with little mesoblast between. There is not sufficient evidence to say whether there is any causal relationship here, i.e., whether the fissure is closed because of the position of the eye or the position of the eye is due to the normal growth of the fissure. The few cases in which the fissure closes in the absence of the lens would suggest that a supplementary factor of some kind is at work that is not strong enough to operate in the majority of cases and can do so only in a few favorable ones and only at a late stage. What these favorable conditions are is hard to guess unless the above-mentioned position of the eye is associated with it. On the other hand, the influence of the lens seems

strong by comparison with this unknown factor, for even in young stages of lens regeneration the choroid fissure begins to behave normally and closes promptly long before the second factor has any effect in the few cases in which it is evident.

Origin of regenerated lenses. Evidently, if a lens is going to regenerate it does so between the stages of 36 and 39. A fully formed young lens is often present in stage 39. Older embryos show free lenses grown to nearly normal size. No young lenses or just forming lenses are present in the older stages. What conditions are favorable for the formation of a new lens from the dorsal rim of the iris has not been determined. Neither the bulging or folding of the middle of the retina nor the collapse of the optic cup is inhibitive, for lenses are found in all of these. No regenerated lenses were found, however, in those forms in which the dorsal anterior border of the optic cup has grown enormously and pushed ventroposteriorly. This excessive growth brings the tapetal layer toward the ectoblast and the dorsal lip is often folded well into the cup. This suggests that a lens can form only when the free rim of the cup is normally placed in regard to the ectoblast. It was suggested by Spemann ('05) that lenses regenerate from the iris when mesoblast is present between the dorsal edge of the cup and the ectoblast. While it is true that mesoblast is present here when lenses have regenerated, it is also true in many cases where no lens is developed. It seems, then, that there can be no causal relation here.

The origin of the lens from the iris in Amblystoma punctatum is in accord with findings in other amphibians. It adds but another form to the list in which such regeneration takes place, the manner of its origin differing in no essential from that described by many workers. As early as 1891 Colucci described and figured such an origin in Triton. Wolff ('95), Müller ('96), Spemann ('05), and Wachs ('14) confirmed this in Triton. Other amphibians in which a similar regeneration of a lens from the rim of the iris have been reported are Rana fusca, Kochs ('97) and Alberti ('22); Bombinator, Kochs ('97); Salamandra maculosa, Fischel ('98, '00, '02); salaman-

drina and axolotl, Wachs ('14); Diemyctylus, Ogawa ('21). Other forms include fishes, Grochmalicki ('08), Alberti ('23), and chick embryos, Barfurth ('02).

With the exception of Spemann's ('05) work, the operation was performed much later than in my experiments, i.e., when the lens was completely formed and was removed through a corneal slit. My results differ in that the lens was never present, the lens anlage having been removed long before there was any evidence of a lens. The temporary presence, then, of a normally formed lens can have no influence in causing the regeneration from the iris rim.

My results throw little further light on the causes which locate lens regeneration on the dorsal rim of the iris. Müller ('96) suggested gravity as the cause. Fischel ('98) eliminates both gravity and light as causes. He feels at this time that the absence of the old lens is sufficient stimulus to explain their appearance. In 1902, he suggests that they are due to injury to the iris in removing the old lens. In 1916, Fischel suggests, in addition to injury, a chemical stimulus arising from a secretion of the retina. Schimkewitsch ('02) suggests an atavistic character to the phenomenon, i.e., he compares the optic vesicle with the structure of the unpaired eye, such as that of Hatteria, in which the lens is formed in the outer wall of the vesicle. This region is suppressed when in ontogeny the optic cup is invaginated, only to reappear when the new lens is extirpated. Wachs ('14), by extremely careful technique in removing the old lens, feels that any injury caused by extirpation is so slight that it cannot be considered a sufficient stimulus to account for the regeneration of the lens from the iris. He further thinks that the stimulus cannot be purely chemical, for he found when a young lens was put into an older eye in which the lens had been removed, regeneration of a new lens was not prevented unless the young transplanted lens lay exactly in the pupil. If it was in the cavity of the cup a new lens developed from the rim of the iris. Again, he thinks pressure from the transplanted lens not a sufficient cause because of certain cases in which a

lens was absorbed, pressure having been exerted on the iris for a time, yet in one case he found a new lens just as far developed as in cases in which no lens was present. He concludes, however, that, in general, if a lens is present there is retardation of regeneration in which secretion and pressure may both be involved. Werber ('18) says: "Undoubtedly with the present data at hand, it is difficult to deny that some chemical process is apparent in the morphogenesis of both the primary and secondary lens. This process, however, may . . . . be due to a substance (a 'lentogenic enzyme') apparently contained by all parts of the eye cup already in the stage of the optic vesicle or even earlier." Yet he feels that "The activation . . . . of this 'enzyme' seems in the case of the 'regeneration' of the lens to be in some way due to the injury of those cells of the iris which subsequently becomes transformed into lens fibers."

Certain it is that in Amblystoma punctatum some special conditions must be responsible for the regeneration of a lens from the iris, since only twenty-four out of the ninety-six embryos studied formed such lenses. The mere absence of the normal lens, implying what it may mechanically or chemically, cannot be the complete cause. Again, very little injury can be caused the optic vesicle in removing the lens-forming ectoblast overlying it in this very early stage, before the invagination of the optic cup and before the formation of the lens. Such slight injury can hardly be sufficient to cause the regeneration of a lens from the iris. Or, granting such an injury, it is hardly conceivable that it would always occur on the dorsal rim of the iris, the place where all lenses regenerated in the embryos of Amblystoma punctatum. Whether the position of the cup after the operation, special types of folding of the collapsed cup, or the presence of mesoblast between the cup and the surface ectoblast are influential is uncertain.

Origin of vitreous humor. A further question as to the influence the lens may have on the normal development of the rest of the eye is raised by experiments involving the absence

of the lens. In all the eyes of Amblystoma punctatum in which the lens is not present the optic cup is variously folded or collapsed and the vitreous humor is absent. The literature shows that this is true for all forms that have been worked on. For example, Lewis ('04) refers to the absence of the vitreous humor in the frog as the cause of "the unexpanded condition of the eye." The point raised is then whether the presence of the lens is necessary for the development of the vitreous humor.

The origin of the vitreous humor has been much discussed and the question has not been settled in any clear-cut fashion. Many of the early workers considered it of mesodermal origin. Later, the discussion centered around its origin from the ectoblast, especially from the retina and lens. In 1898, Tornatola described the vitreous humor as arising in birds and mammals (including man) from the retina by means of cone-shaped processes later extending into fibrils which eventually form an anastomosing network in the optic cavity. This point of view is supported by Fischel ('00) in the salamander; Retzius ('94) in the frog; Rabl ('03) in fishes and Amphibia; Kölliker ('03) in birds and mammals.[6] Lenhossék ('03) describes the vitreous humor in mammals and man as developing in the same manner by means of protoplasmic processes from the lens only and denies the participation of the retina in its formation. Cirincione ('03) finds the vitreous humor developing in the same way by means of processes extending into fibrils which take their origin from both the retina and the lens. Van Pée ('03), on the other hand, in his study of mammals, thinks that not only the retina and lens contribute fibers, but that the mesenchyme plays an important part. The fibrils derived from the lens he describes as transitory, since they form the lens capsule and take no part in the formation of the humor. The mesen-

[6] Kölliker recognizes the participation of the mesoblast during development referring to it as the "Mesodermaler Glaskörper" but says: "Da die Glaskörper- und Linsengefässe später schwinden, so kann beim ausgebildeten Auge nicht mehr von einem mesodermalen Glaskörper gesprochen werden und ist der reife Glaskörper als wesentlich ektodermale, d.h. retinale Bildung anzusehen."

chyme cells which enter with the vascular tissue form concentric fibers in the part of the cup nearest the lens, while the fibers which arise from the retina are radial and lie at right angles to the mesodermal fibers, thus forming a network. This theory of the origin of the vitreous humor from the retina, lens, and mesenchyme is supported by von Szily ('04) who worked on the trout and the chick. He stresses the similarity of the vitreous humor with the general connective tissue which he claims is first formed by fibrils given off from all epithelial tissue, whatever the germ layer to which it belongs. He states that later these fibers join the mesenchyme cells, forming a new union, while the original connection is lost. Thus, in the case of the vitreous humor, fibers arise from both the retina and the lens which later join the mesenchyme cells which have invaded the cup with the vascular tissue. This, he feels, answers the criticism of all those workers who deny the participation of the lens in the formation of the vitreous humor on the ground that fibers are given off from the front of the lens as well as the inner surface and that therefore fibers from the lens can have no meaning in the formation of the vitreous humor. If all epithelial tissue contributes fibers as preliminary to the formation of general connective tissue, it would, he feels, be normal to find processes and fibers given off from the front of the lens for this purpose and from the inner surface to help form the vitreous humor. A pupil of Lenhossék, von Szily is emphatic in his support of the part played by the lens in the formation of the vitreous humor.

Further emphasis is placed on the rôle played by the mesodermal element by Froriep ('05), who states that, while the primitive vitreous humor is ectodermal (retinal) in origin, the character of the definitive vitreous humor is determined not by the exceedingly delicate and perishable ectodermal portion, but by the strongly developed mesodermal constituents.

The double origin of the vitreous humor from mesoderm and ectoderm seems to be well established for mammals. The

fact that there are but few mesenchyme cells present in the optic cup of the lower vertebrates (fishes and amphibia), as well as in the chick, makes it seem less certain that they play an important part in the formation of the humor in these forms, although von Szily feels that he has demonstrated their presence in small numbers and the union of the fibrils with them in both the fish and the bird. The part played by the lens is equally uncertain. If von Szily is right as regards the connective-tissue nature of the vitreous humor, one must accept the lens as a factor in the development of the vitreous humor.

The fact that, in the experiments on Amblystoma punctatum reported in this paper, the vitreous humor is not formed in the absence of the lens and the eye is either collapsed or folded suggests that the lens plays an important part in the normal development of the humor. My sections were not preserved and stained with this point in view, and no other investigator seems to have reported on the origin of the vitreous humor in Amblystoma. That the lens may be a factor is evident from the literature quoted for other forms, including all classes of vertebrates. Further experiments on Amblystoma with special reference to the development of the optic cup and the vitreous humor in the absence of the lens may help determine the exact rôle of the lens in the formation of the vitreous humor.

SUMMARY

1. In the embryo of Amblystoma punctatum, when the lens-forming ectoblast is removed in the neural-fold stage and replaced by foreign ectoblast, no lens is formed from this graft. In the absence of the lens, in all but a few exceptional cases, the choroid fissure fails to close during the later development of the eye, but remains open, the optic nerve running along the open groove to the brain. The presence of the lens, then, is apparently an important factor for the normal development of the choroid fissure and the optic cup.

2. In a few of the older embryos, in the absence of the lens, a late adjustment has taken place, so that the choroid fissure is closed. The optic nerve may come off from the eyeball too far ventrally, suggesting a late closure. Some influence other than the presence of the lens is present in these forms. It is effective only in a late stage of development and is not sufficiently strong to replace the influence of the lens in more than a few cases.

3. In about 25 per cent of the embryos studied a lens regenerated from the dorsal rim of the optic cup (iris) in a way practically identical with that process described in other amphibians.

4. In all embryos in which a lens was formed or was forming in this way from the iris, the choroid fissure was closed normally or closing if the lens anlage was very young. The eye was normal in position and structure. This further confirms the existence of a causal relation between the presence of the lens and the ability of the choroid fissure to complete its closure.

5. Lenses of this kind were not developed in embryos in which the optic cup showed a dorsal anterior thickening, thus bringing the pupil ventroposterior in position, or in forms in which there is an eversion of the whole cup. They did appear in eyes in which the pupil was lateral or ventrolateral in position whatever the type of modification present in the eye.

6. That the choroid fissure is not specific and localized is shown by a series of experiments in which the whole optic vesicle was rotated 180°, since the choroid fissure appeared in every case on the new ventral side (original dorsal) of the optic cup. This was true whether or not a lens was present.

7. Since the vitreous humor fails to form in eyes in which the lens is absent and the optic cup is collapsed or folded, these experiments may throw some light on the debated question of the participation of the lens in the development of the vitreous humor.

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PLATE 1

EXPLANATION OF FIGURES

All figures of Amblystoma punctatum

19 Cross-section through the primitive forebrain (stage 21), showing the optic vesicle as a wide-open evagination just touching the surface ectoderm. This stage, shown in figure 1, was most frequently used in operations. × 60.

20 Cross-section through the right (operated) eye of a young embryo (stage 31), in which the lens-forming ectoblast has been replaced by foreign ectoblast. × 60.

21 Cross-section of left (normal) eye of the same embryo as 20. No lens has yet formed. × 60.

22 Cross-section of the right (operated) eye of an embryo (stage 37), showing wide-open choroid fissure. Figure 2 shows a reconstruction of this eye. × 60.

23 Cross-section of the left (normal) eye of the same embryo as 22, showing lens well formed and choroid fissure closed. × 60.

24 Frontal section through the ventral part of the right (operated) eye of a much older stage (42), showing the posteroventral position of the pupil, the excessive anterior growth, and the failure of the choroid fissure to close. Figure 3 gives a reconstruction of this eye. × 60.

25 and 26 Two sections through the right (operated) eye of a young embryo (stage 37), showing the excessive dorsal and anterior thickening and folding of the optic cup and the open choroid fissure. Figure 4 is a reconstruction of this eye. × 60.

27 Frontal section of the right (operated) eye of an older embryo than the last, showing the posteroventral position of the pupil, excessive anterior growth, and incomplete choroid fissure. Figure 5 is a reconstruction of this eye. × 60.

For list of abbreviations see page 221.

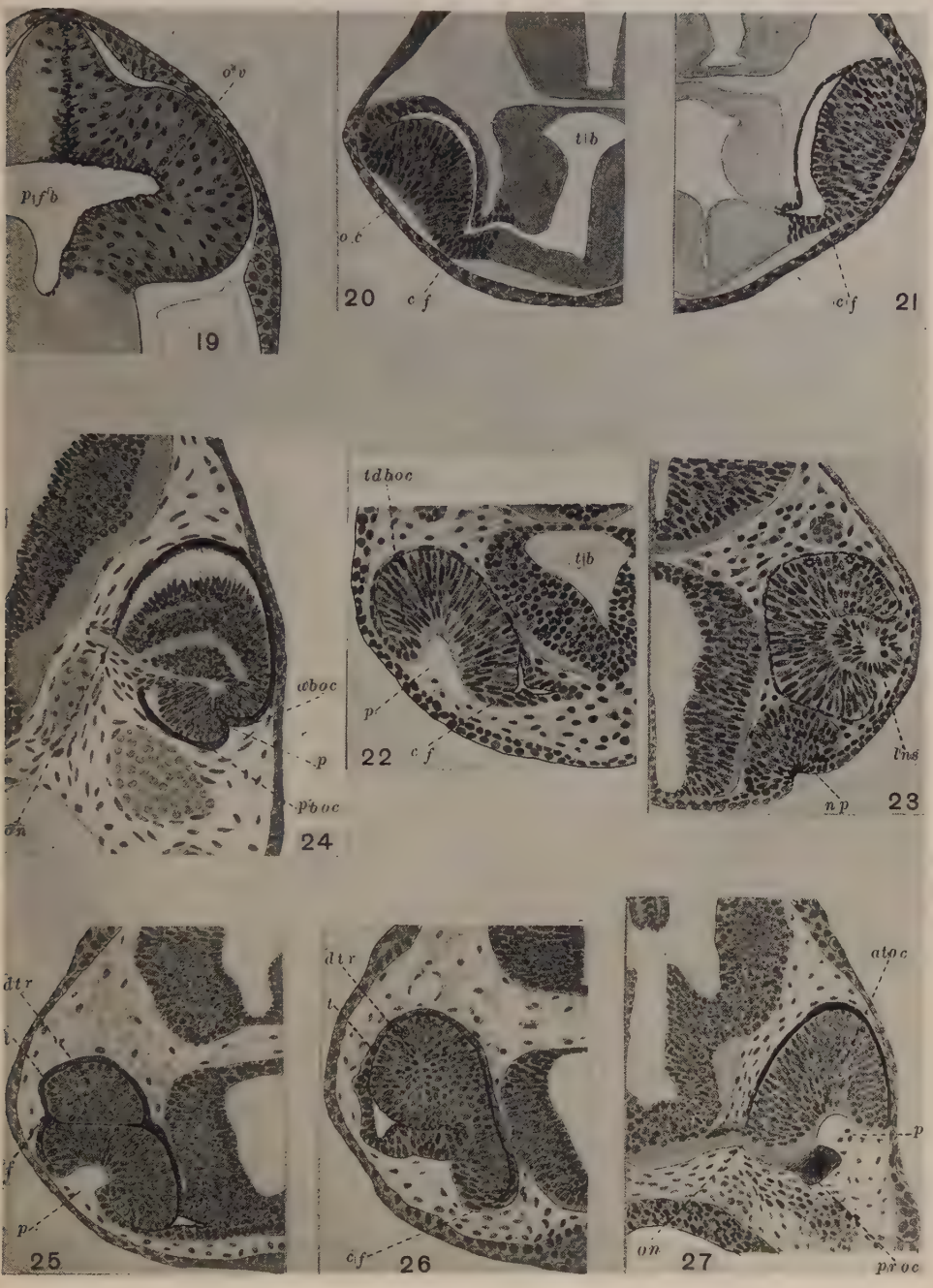
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PLATE 2

EXPLANATION OF FIGURES

All figures of Amblystoma punctatum

28 Cross-section through the right (operated) eye of an embryo (stage 38), showing excessive downward growth of the dorsal border of the optic cup accompanied by the failure of the choroid fissure to close. Figure 6 is a reconstruction of this eye. × 60.

29 Cross-section of the right (operated) eye of an older embryo (stage 42), showing a later stage of the excessive downward growth of the dorsal border of the optic cup. Section just in front of the choroid fissure. × 60.

30 Cross-section of the left (normal) eye of the same embryo just in front of the optic nerve. × 60.

31 Cross-section of the right (operated) eye of an embryo (stage 40) in which the base of the optic cup is thickened and bulges into the pupil. Figure 8 is a surface view of this embryo. × 60.

32 Cross-section through the right (operated) eye of an older embryo (stage 45), showing the eversion of the whole retina into the wide-open pupil. Figure 9 is a surface view of this embryo. × 55.

33 Cross-section of the same embryo just back of the median plane, showing the open choroid fissure. × 55.

34 Cross-section through the anterior third of the right (operated) eye of a still older embryo (stage 46). Similar to the above, but not everted so strongly so that the pupil is small and lateral in position and filled with a retinal plug. Figure 10 is a surface view of this embryo. × 55.

35 Cross-section of a young stage (stage 39), showing complete eversion of the optic cup leaving no iris rim. Figure 11 is a surface view of this embryo. × 55.

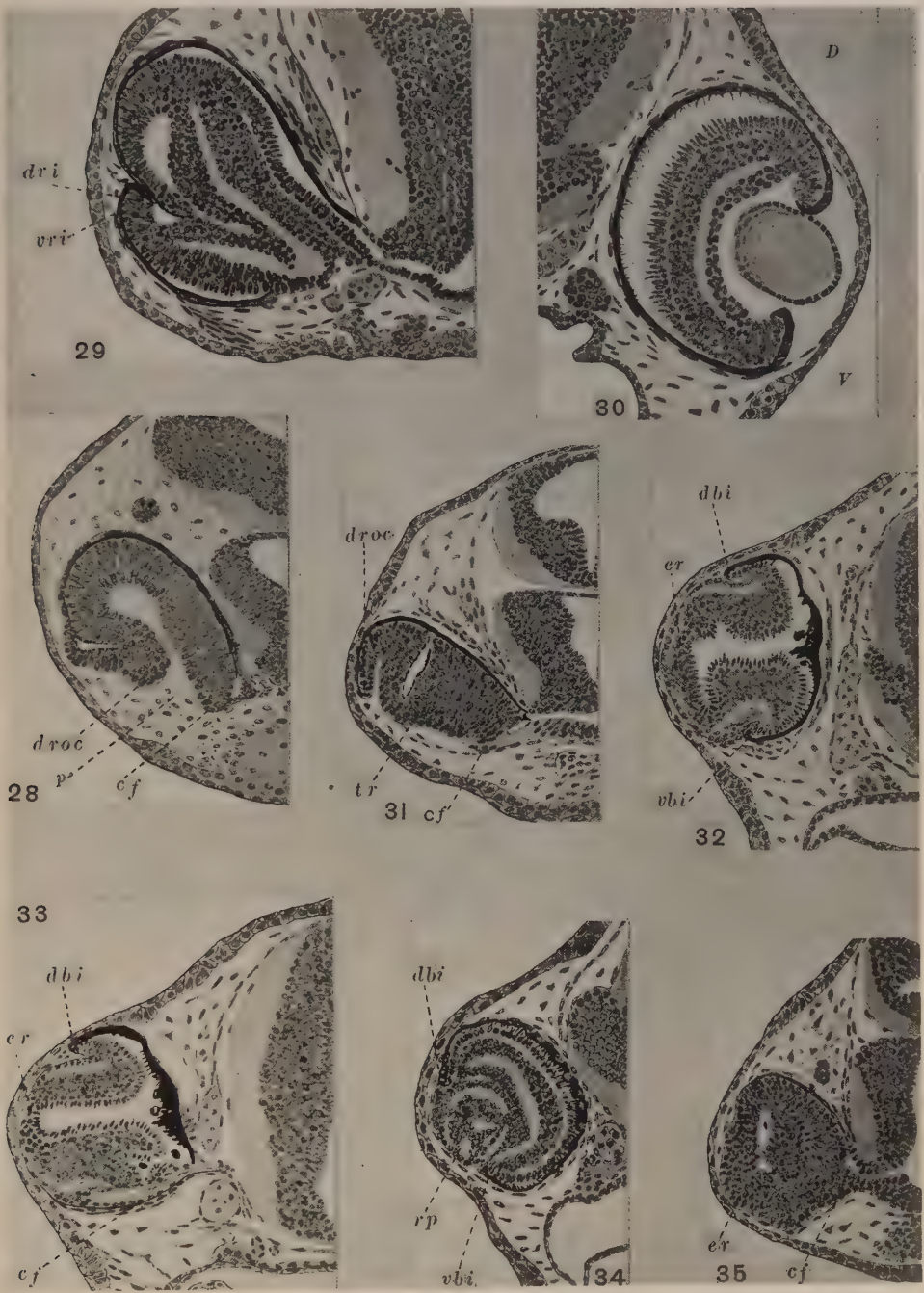
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PLATE 3

EXPLANATION OF FIGURES

All figures of Amblystoma punctatum

36 Cross-section of an older embryo (stage 45), showing complete eversion of the optic cup with a small dorsal iris rim and an open choroid fissure. Figure 12 is a surface view of this embryo. × 55.

37 Cross-section of a late embryo (stage 44), showing a retinal plug filling the pupil and a closed choroid fissure. The position of the pupil is nearly normal. See figure 13. × 55.

38 Cross-section of a late embryo (stage 47), showing a collapsed optic cup, completely closed choroid fissure and normal position of the pupil. See figure 14. × 55.

39 Cross-section of the dorsal border of the iris through the anterior portion of the right (operated) eye of an embryo (stage 39), showing the early stage in the regeneration of a lens from the iris rim. Surface view shown in figure 15. × 370.

40 Cross-section through the posterior region of the same eye as 39 back of the lens anlage, showing that the choroid fissure has begun to close regularly with no folding or thickening of the wall of the cup. × 55.

41 Cross-section of a slightly later stage in the regeneration of a lens from the iris rim (stage 40), showing the beginning of the cutting off of the lens from the dorsal border of the optic cup and the radial arrangement of the cells. × 370.

42 Cross-section of a still older stage in the regeneration of a lens from the rim of the iris (stage 39), showing the lens-like structure and the narrow neck of attachment to the tapetal and retinal layers. Figure 16 is a surface view. × 370.

43 Cross-section through the posterior region of the same eye as 42, showing that the choroid fissure is completely closed in the presence of a young regenerated lens. Surface view shown in figure 16. × 55.

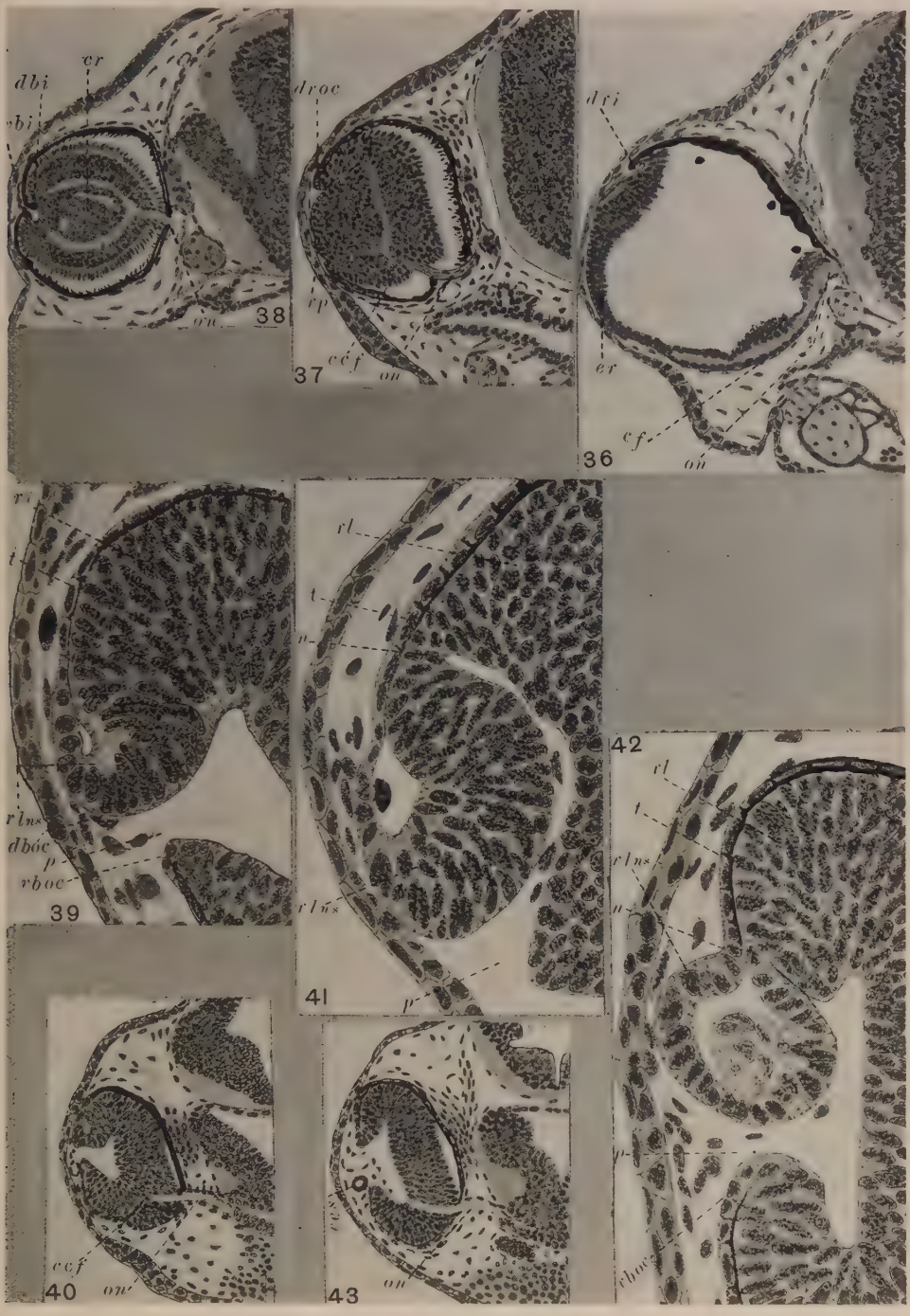
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PLATE 4

EXPLANATION OF FIGURES

All figures of Amblystoma punctatum

44 Cross-section through the eye of an embryo (stage 39), showing a small regenerated lens completely free, but pressed against the upper rim of the iris and occupying but one-half of the pupil opening. This section is through the region of the choroid fissure, which is completely closed. × 55.

45 Cross-section of a slightly older regenerated lens (stage 43), showing the rapid differentiation of the typical lens structure and rapid growth, though still incompletely filling the pupil and pressed against the dorsal rim of the iris. × 55.

46 Enlarged portion of the dorsal rim of the iris of the same eye as in 45, showing the lens pressing against the iris, which is flattened. × 370.

47 Cross-section of an embryo (stage 41), showing a completely regenerated lens present in an eye in which the retina is folded so as to push out into the pupil. The lens is dorsal, distorted, and pressed in between the dorsal rim of the iris and the retinal fold which occupies the ventral portion of the pupil. × 55.

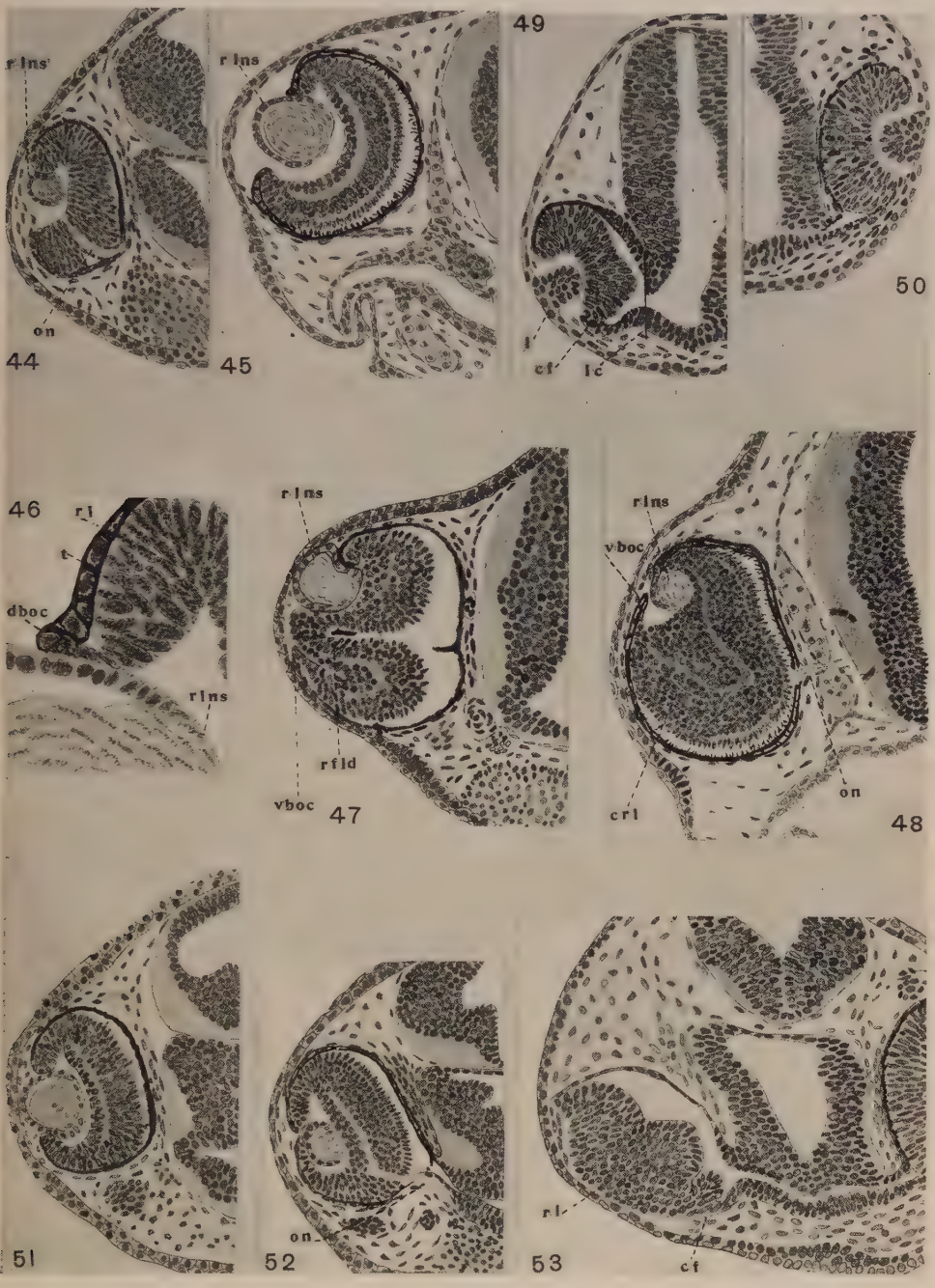
48 Cross-section of an embryo (stage 46), showing a regenerated lens in the collapsed type of eye. The lens is pressed against the dorsal rim of the iris in which it is partially embedded, while the outer layer is still continuous with the tapetal layer. × 55.

49 Cross-section through the right eye of an embryo (stage 36) which, together with the surface ectoblast, was rotated 180° in the optic-vesicle stage (fig. 19), showing the new normal-appearing union which the optic cup has made with the brain and the choroid fissure present in the normal ventral position, developed from the original dorsal portion of the cup. × 55.

50 Cross-section of the left (normal) eye of the same embryo as figure 49. × 55.

51 and 52 Cross-sections of an older embryo (stage 40) through the right eye which, together with the surface ectoblast, was rotated 180° in the optic-vesicle stage, showing the normal character of the eye. Figure 51 is through the choroid fissure region, showing its presence in the normal ventral position, but developed out of the original dorsal side of the cup. Figure 17 shows this embryo in surface view. × 55.

53 Cross-section through the head of an embryo (stage 37) in which the right optic vesicle was reversed 180° in stage 21 and the lens-forming ectoblast replaced by a graft of foreign ectoblast. No lens is present, the retinal layer is thickened and everted so that no pupil is formed, and the choroid fissure is developed out of the original dorsal portion of the optic cup. Figure 18 shows the surface view of this embryo. × 55.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THE RATE OF OXYGEN CONSUMPTION IN FIVE SPECIES OF AMBLYSTOMA LARVAE [1]

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INTRODUCTION

The occurrence of variations in the growth rates of homoplastic and heteroplastic grafts in Amphibia has been noted by workers in this field from time to time. Recently, Harrison ('24) described the striking effect on the size and growth rate of fore limbs transplanted in embryonic stages from A. punctatum to A. tigrinum and vice versa. The punctatum limbs, grafted to tigrinum larvae, developed into appendages which were small as compared with the normal tigrinum and punctatum fore limbs. The reciprocal graft (tigrinum limb to punctatum larvae) resulted in an abnormally rapid growth and development of the transplanted fore limb, which greatly exceeded in size both the normal tigrinum and punctatum fore limbs. In view of these results and of similar grafting experiments made with other organs, such as an eye, the suggestion was made by Doctor Harrison that a determination of the relative metabolic rates of various species of Amblystoma larvae might throw some light on the factors involved in the production of such abnormalities, and indirectly on the factors which are concerned in the growth and development of the normal limbs.

The determination of the metabolic rates of A. tigrinum larvae and of young axolotls seemed desirable for a second reason, namely: if a distinct difference should be found to

[1] The writer wishes to express his appreciation of the kindness of Dr. R. G. Harrison, of this laboratory, in supplying the various stocks of Amblystoma larvae used in the present investigation.

exist, some clue might be suggested as to the factors controlling the peculiar neotenous condition of the latter species. If the rate of metabolism of the axolotl is in any way responsible for its abnormally prolonged larval life, we should expect this rate to be considerably lower than that of A. tigrinum.

Oxygen consumption, which is one phase in developmental metabolism, was determined for five species of Amblystoma larvae, all of which have been used by Doctor Harrison in heteroplastic-grafting experiments. The following series of experiments were performed:

1. T series (ten cases): A. tigrinum larvae.
2. P series (ten cases): A. punctatum larvae.
3. J series (ten cases): A. jeffersonianum larvae.
4. M series (ten cases): A. microstomum larvae.
5. T′ series (twelve cases): A. tigrinum larvae.
6. P′ series (twelve cases): A. punctatum larvae.
7. TA series (seventeen cases): A. tigrinum larvae (axolotl).

MATERIALS AND METHODS

The determination of the oxygen consumption of all species tested was ascertained by the Winkler method, which is sufficiently reliable to give a fairly accurate estimate of the rate of oxidations in the organism. It can be relied upon, particularly in comparative work, and especially where considerable differences in oxidative rate exist.

The animals tested were in all cases starved for twenty-four hours preceding the test and placed in water which was maintained at 20°C. This temperature was also used during the testing period for all species. It was found undesirable to anaesthetize specimens during the period of confinement in the testing flasks, and so any possible change in the normal oxidative rate due to anaesthesia was avoided. The animals remained quiet during most of the four-hour testing period and data from animals exhibiting excessive activity were discarded. As a matter of fact, the species exhibiting the high-

est metabolic rate were those which were the least active in the testing flasks, which fact would tend to emphasize the species differences that were found to exist. All animals were tested for four hours and weighed immediately afterward, in order to calculate the oxygen consumption per gram weight per four hours. In the cases of series T, P, J, and M, all individuals were tested twice and the average consumption computed from the data obtained.

The A. punctatum and A. jeffersonianum larvae were hatched from eggs obtained in the vicinity of New Haven, Connecticut; the axolotls from eggs laid in the laboratory by individuals imported from Germany, and the A. microstomum and A. tigrinum from eggs obtained from Urbana, Illinois, through the kindness of Prof. Charles Zeleny. All specimens used, however, had been reared under laboratory conditions and fed the same diet. The individuals tested exhibited approximately the same developmental stage, weight, and body dimensions. Variations in the metabolic rate due to such causes were thereby eliminated, which was essential in considering possible differences in metabolism between species All specimens were approximately two months old and varied between 30 and 35 mm. in length. The fore limbs averaged 5 mm. in length and the hind limbs 4 mm.

Series T, P, J, and M

The mean oxygen consumption per gram weight per four hours, for these four series, together with related data, will be found in table 1. Series T (A. tigrinum) gave the lowest oxygen consumption of the four species tested, or .4447 $\pm$ .0147 cc. of oxygen, while series M (A. microstomum) was remarkably high, being 1.3339 $\pm$ .0502 cc. of oxygen. Series J (A. jeffersonianum) and series P (A. punctatum) were intermediate, giving .5268 $\pm$ .0363 and .6531 $\pm$ .0265 cc. of oxygen, respectively. Comparing the tigrinum and punctatum series, we find that the latter is approximately 46.9 per cent higher than the former in oxygen consumption—a difference not to be attributed to random sampling, in view of the

fact that the difference between the respective means was approximately seven times the probable error of the difference. Series J (A. jeffersonianum), on the other hand, although exhibiting a 24 per cent lower oxygen consumption than series P and an 18.5 per cent higher oxygen consumption than series T, is nevertheless doubtful, due to the fact that the differences exceeded the probable errors of the differences

TABLE 1

Oxygen consumption of four species of Amblystoma larvae per gram weight, four hours. Age, two months

SPECIES	MEAN	STANDARD DEVIATION	NUMBER OF ANIMALS TESTED	AVERAGE GRAM WEIGHT
I (A. tigrinum)	.4447 ± .0147	.0687 ± .0104	10	.250
II (A. punctatum)	.6531 ± .0265	.1244 ± .0121	10	.202
III (A. jeffersonianum)	.5268 ± .0368	.1728 ± .0261	10	.241
IV (A. microstomum)	1.3339 ± .0502	.2354 ± .0355	10	.175

SPECIES COMPARED	DIFFERENCES BETWEEN MEANS	DIFFERENCE / P.E. DIFFERENCE	AVERAGE PER CENT EXCESS OF ONE SPECIES OVER ANOTHER
Punctatum—Jeffersonianum	.1263 ± .0454	2.78	24
Punctatum—Tigrinum	.2084 ± .0303	6.88	46.9
Jeffersonianum—Tigrinum	.0821 ± .0396	2.08	18.5
Microstomum—Tigrinum	.8892 ± .0523	17.00	200
Microstomum—Jeffersoniaum	.8071 ± .0622	12.81	153.2
Microstomum—Punctatum	.6808 ± .0568	11.99	104.2

only by about two and three times, respectively. It seems probable, however, that the per cent differences recorded are accurate within 5 per cent, at least, and are not due to the effects of random sampling. Series M (A. microstomum) was by far the highest in oxygen consumption, being 104 per cent higher than the punctatum series, 153.2 per cent higher than the jeffersonianum series, and 200 per cent higher than the tigrinum series. The large ratios of the differences of the means to their probable errors (12, 13, and 17, approxi-

mately) leave no doubt that a great difference in oxygen consumption exists between this species and the other three tested.

Series T′, P′, and TA

The animals used in series T′ (A. tigrinum), series P′ (A punctatum), and series TA (A. tigrinum, axolotl), were animals hatching one year after those used in the first four series. The punctatum series again showed an increase of

TABLE 2

Oxygen consumption of Amblystoma larvae (tigrinum, punctatum, and axolotl) per gram weight, four hours. Age, two months

SPECIES	MEAN	STANDARD DEVIATION	NUMBER OF ANIMALS TESTED	AVERAGE GRAM WEIGHT
A (A. tigrinum)	.6434 ± .0464	.2385 ± .0328	12	.313
B (A. punctatum)	.9472 ± .0519	.2665 ± .0367	12	.214
C (Axolotl)	.6656 ± .0236	.1440 ± .0167	17	.300

SPECIES COMPARED	DIFFERENCES BETWEEN MEANS	DIFFERENCE / P.E. DIFFERENCE	AVERAGE PER CENT EXCESS OF ONE SPECIES OVER ANOTHER
Punctatum—Tigrinum	.3038 ± .0696	4.37	47
Punctatum—Axolotl	.2816 ± .0570	4.94	42
Axolotl—Tigrinum	.0222 ± .0521	.43	3

approximately the same amount, or 47 per cent (table 2), over the tigrinum series. The ratio of their difference to the probable error of their difference, although but a little over 4, is sufficient to justify the per cent difference recorded as an actual species difference. The axolotl series (series TA) closely approximated the tigrinum series, being but 3 per cent higher than the latter, which difference is insignificant, in view of the ratio of the difference of the means of the two series concerned to the probable error of the latter. Compared with the punctatum series, however, it gives, like the

tigrinum series, a marked difference, or, to be exact, 42 per cent.

DISCUSSION

In comparing the means of series T and P with those of series T′ and P′, respectively, it will be noticed that those of the latter series are 50 per cent higher, approximately. This difference is due, no doubt, to a difference in stocks. The significance of the species differences determined, therefore, is not lessened in any way. It is possible that the various species tested possess different degrees of respiratory regulation under different oxygen tensions. If this were so, the exact quantitative differences would be less accurate than recorded. It is the writer's opinion, however, that the possibility of thus affecting the relative order of the species differences is very slight.

The question naturally suggests itself as to whether or not the proximity of metamorphosis could be used as an explanation of the differences in metabolic rate which were found to be present. According to Abelin and Scheinfinkel ('23), the metabolic rate of artificially metamorphosed axolotl (Tigrinum mexicanum) is lowered from 50 to 70 per cent below the normal larval rate during metamorphosis. The same authors likewise found that the metabolic rate of Rana esculenta larvae decreased during metamorphosis, while Groebbels ('22) records the same to be true of Rana temporaria larvae, basing his conclusions on their lowered oxygen consumption. The writer ('23) obtained very different results, however, with Rana pipiens larvae. It was found that a progressive rise in oxygen consumption per gram weight took place as the tadpoles approached the end of metamorphosis, amounting to 79 per cent above the normal larval consumption toward the end of their transformation.

As regards the Amblystoma larvae used in the present investigation, metamorphosis could not have been concerned in any way with the variations found to exist between the various species or members of the same species. In other words, the animals were tested at a time considerably prior

to the onset of metamorphosis. Representative members of series P, J, M, and T were maintained under constant environmental conditions following the testing period, and were found to exhibit the first signs of metamorphosis approximately four, four and a half, four and a half, and five months later, respectively. There was also no evidence that individuals whose metabolic rate was lower than that of other members of the same species underwent metamorphosis at an earlier age than the latter.

The results obtained with the Amblystoma larvae, as described in this paper, clearly indicate that considerable differences of metabolic rate exist between the various species tested, as they probably do between the larvae of most amphibians. This fact is of interest and suggestive when considering the different growth rates of heteroplastic grafts. For example, if a heteroplastic graft were transplanted from a species of comparatively low metabolic rate to one of a comparatively much higher metabolic rate, we should expect the graft to grow more rapidly than would have been the case if not removed from the donor. Were we to consider only the combinations between A. punctatum, on the one hand, and A. tigrinum and A. mexicanum, on the other, the parallel between difference in rate of oxygen consumption and the effect of heteroplastic grafting is striking. Unfortunately, however, when extended to the other species in question, the correlation breaks down. The results of the transplantation experiments in which A. jeffersonianum and A. microstomum were used are as yet unpublished, but Doctor Harrison informs me that jeffersonianum, with an oxygen-consumption rate midway between punctatum and tigrinum and microstomum, which has a much higher rate than any of the other forms, behave in respect to heteroplastic grafting in essentially the same way as punctatum, i.e., the tigrinum limb is accelerated when grafted to jeffersonianum, and the microstomum and jeffersonianum limbs are retarded when grown on tigrinum larvae. Furthermore, all of the combinations between punctatum, jeffersonianum, and microstomum show

an essentially neutral effect on growth rate, although the oxygen-consumption rates are very different. Doctor Harrison suggests, as a possible explanation, that the oxygen-consumption rate in jeffersonianum may be sufficient to produce the maximum possible effect. At any rate, the facts given above are of interest and merit further investigation.

The close agreement of A. tigrinum larvae and axolotl larvae, as regards their oxygen consumption, is interesting to consider at this time. While the axolotl is thought by many to be a distinct species of Amblystoma, others regard it as merely a strain of A. tigrinum, in which the individual remains indefinitely in a neotenous condition. The fact that they are larvae whose period of metamorphosis is greatly delayed, would lead one to expect a lowered metabolic rate. The results obtained, however, indicate that there is apparently no difference in this respect between the two strains, and, in fact, the axolotl tested averaged a 3 per cent higher metabolic rate than the tigrinum tested. This difference is statistically insignificant, however, for the probable error of the difference of the means was approximately double their difference. The metabolic rates, therefore, of axolotls cannot be used in any way as an explanation of their neotenous condition.

SUMMARY AND CONCLUSIONS

1. The oxygen consumption per gram weight in four hours, of five species of Amblystoma larvae, was determined by means of the Winkler method and distinct species variations found to exist.

2. The mean oxygen consumption of ten A. microstomum larvae was 200 per cent higher than the mean consumption of ten A. tigrinum larvae, 153.2 per cent higher than the mean consumption of ten A. jeffersonianum larvae, and 104.2 per cent higher than the mean consumption of ten A. punctatum larvae.

3. A second series of twelve A. punctatum and twelve A. tigrinum larvae emphasized the results previously obtained,

that the former species exhibit approximately a 47 per cent higher oxygen consumption than the latter species.

4. The results obtained indicate that the metabolic rates of different species of Amblystoma larvae are widely divergent and may possibly be one of the factors involved in differential growth rates, especially where heteroplastic grafts are concerned. The rather wide individual variations between members of the same species could likewise be applied in interpreting the growth rates of homoplastic grafts.

5. Seventeen axolotl (A. tigrinum) gave approximately the same oxygen consumption as was found in the case of normal A. tigrinum larvae. This fact indicates that the neotenous condition of the axolotl cannot be interpreted in the light of a supposed difference in metabolic rate, as compared with normal A. tigrinum larvae.

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THE EFFECT OF SELECTION IN PEDIGREE LINES OF INFUSORIA[1]

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SIX FIGURES

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INTRODUCTION

Since Johannsen formulated the concept of the genotype, a great number of investigators have devoted much study to the problem of variation, inheritance, and the effects of selection within the progeny of a single individual where there is no mixing of diverse lines of descent. However, at the present time there seems to be no explanation which serves to interpret the apparently diverse results obtained by the various workers.

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After making his well-known tests on self-fertilized beans and barley, Johannsen ('03) showed that many pure lines, very slightly differentiated from each other, exist in nature, but that selection within a pure line is absolutely without effect in modifying a particular character in question.

The phenomenon of complete regression in such material has been shown by a great number of other investigators to be of very general occurrence. Essentially the same state of affairs exists, not only in self-fertilized plants, but also in plants reproducing vegetatively, by tubers or cuttings, and in animals reproducing by parthenogenesis, budding and fission. For all forms of inbreeding, resulting in the production of homozygous individuals, are concerned with nearly the same situation. In all cases there is no mingling of genotypes of diverse genetic constitution.

Although we shall confine our discussion to the Protozoa, it will not be possible to make even a general survey of the literature which is devoted to the study of the effect of selection in uniparental reproduction; this has been done many times, and very completely, by previous authors. For an excellent review of the problem, the reader is referred to Jennings ('20). However, brief mention may be made of a few investigations which have a more direct bearing on the present work.

Jennings ('08), in his extensive work on Paramecium aurelia and Paramecium caudatum, found these species to consist of an assemblage of heritably diverse stocks ('pure lines') which differ from each other by rather definite heritable morphological characters, such as length and breadth of the cell; and also that differentiations which are hereditary do not arise in a line reproducing vegetatively. The variations in the 'pure line' were temporary fluctuations and did not alter the constancy of the genotype. In brief, selection within the 'pure line' had no effect on the morphological characters in question. Ackert ('16) has more recently repeated these experiments, with the same results.

But there are others who have maintained that selection within the clone may have an effect. Weldon and Pearson ('03) and Pearson ('10) have published severe criticisms of the earlier work on which the genotype hypothesis was founded and have subjected, among others, the data of Johannsen to a detailed statistical treatment. These authors showed, from the data of these observers, that, inasmuch as the parental correlation was much higher than that for the more remote ancestry, the individual characteristics of the parents have more influence in determining those of the offspring than have those of ancestors further removed. Furthermore, since regression was shown to take place within the 'pure line,' they maintained that properly instituted selection would produce effect within the limits of the 'pure line.'

The effectiveness of selection in regard to what may be termed a physiological character has been tested by Middleton ('15) in a study of the fission rate of another ciliate, Stylonychia pustulata. This author found, to his surprise, that by opposite selection, through more than 150 generations, of small individual variations occurring among the progeny of a single individual, it was possible to produce two sets differing hereditarily in rate of fission. During this selection there was a gradual increase in the average heritable difference between the two sets, showing that the effect of selection was cumulative. The hereditary differences persisted through periods of balanced selection and through conjugation, and only disappeared under the influence of reversed selection. Middleton therefore concluded that "in Stylonychia, from a single clone of given genotype, it is possible to obtain, through long continued selection during reproduction by fission, two sets (clones?) of diverse genotype, differing characteristically from each other in rate of fission, under identical conditions, and retaining these differences from generation to generation. The selection of small variations, such as appear within the 'pure strain' or clone, is then an effective evolutionary procedure."

Jennings ('16) has demonstrated that in the uniparental reproduction of the rhizopod, Difflugia corona, the number and length of the spines on the test, the diameter and depth of the shell, as well as other morphological characters, become differentiated in the descendants of a single individual; and that by selection it is possible to isolate many strains that differ markedly in their genotypic condition. In this form, unlike Paramecium, he found selection within the progeny of a single individual to be effective. Still more recently, the same methods of work have been applied to two other rhizopods. Root ('18) has recorded considerable evidence that, in Centropyxis aculeata, certain variations within the pure stock were inherited, and a quite similar result has been reached by Hegner ('18) in Arcella dentata.

Erdmann ('20) has analyzed Middleton's results and has suggested that there may be a period in the life of Stylonychia pustulata, reproducing vegetatively, where selection is more effective than in others. This she holds to be true for Paramecium aurelia, in which she has studied the occurrence of size variations with reference to endomixis, and has found that "each time after endomixis different lines with less or greater differences in size appear."

We are thus confronted with a marked diversity of ideas concerning the origin of hereditarily differentiated strains among the descendants by fission of a single protozoon and their isolation by selection.

The problem

In view of the apparent divergence of results obtained from Paramecium with those from Stylonychia and various rhizopods, a series of experiments was carried out which involved the selection of a physiological character in various pure strains of Infusoria, including Paramecium aurelia, Paramecium calkinsi, and Stylonychia pustulata.

Although races have their own peculiar division rate under given conditions and although, in general, there is a remarkable synchronism of fission in closely related cells of a race

under identical conditions, it is found that, as a rule, one cell of two sister cells will divide slightly earlier than the other. This variation in the rate of division of individual cells is the 'physiological character' which has been selected in this work.

Thus, as shown in figure 1, at a given moment we may have among the progeny of a single parent individuals of the second and fourth generations. These differences of fission rate among the descendants of a single parent afford, therefore, an excellent opportunity for selection to determine whether such differences are inherited and whether the progeny of a single parent can be broken up into fast and slow lines which show a significant difference in rate of division.

In the first species, P. aurelia, endomixis has been demonstrated, while in P. calkinsi it has never been observed, and apparently does not occur (Spencer, '24). If the phenomenon of endomixis has any direct bearing on the problem of heredity in the Infusoria, we should detect any such relationship from a study of the effect of selection based upon properly designed experiments using both forms.

The experiment was repeated on the hypotrich, Stylonychia pustulata, for the purpose of comparison with the two holotrichous forms, P. aurelia and P. calkinsi. Since it was necessary to modify somewhat the method used by Middleton ('15), it was deemed necessary to repeat, in part, the experiment using the form which he used in order that the comparison might be more complete.

In brief, the following questions present themselves for solution: 1) In the three species studied, is it possible to isolate by selection hereditarily diverse strains with reference to a physiological character? 2) If the three species do not lend themselves equally to the production of diverse strains by selection, can this difference in behavior be attributed to any known difference in the constitution of the races, e.g., to endomixis? 3) If so, to what extent can this factor be correlated with the results of selection in each race?

This problem was suggested by Prof. Lorande L. Woodruff, to whom the author wishes to express his appreciation for invaluable counsel throughout the investigation. He is also indebted to Dr. Dewey G. Steele for many helpful suggestions with regard to the statistical treatment of the material.

MATERIALS AND METHODS

The race of Paramecium aurelia employed in this study was derived from a single animal from Woodruff's pedigree culture on July 26, 1925, then in approximately the 11,000th generation. The Paramecium calkinsi were descended from a race collected during August, 1924, at Woods Hole, Massachusetts, and the Stylonychia pustulata were also obtained at Woods Hole in July, 1926.

The organisms were bred on depression slides which, as usual, were kept in moist chambers to prevent evaporation of the culture medium. Isolations and transfers were made daily throughout the work. A 'varied culture medium' was employed. Such a medium has been found to be the most satisfactory for the well-being of pedigree lines (Woodruff, '08). For some experiments the medium consisted of a 0.025 per cent solution of Liebig's beef extract in glass-distilled water (Woodruff and Baitsell, '11), while for others it consisted of varying concentrations of fresh hay infusion made up sometimes with tap-water and sometimes with spring water. In other cases, the medium consisted of a combination of the two, i.e., beef and hay together. In the case of the animals obtained from Woods Hole it was necessary, for the maintenance of life, to make the culture medium slightly saline by the addition of a very small quantity of a 0.75 per cent solution of sodium chloride to the medium. The bacterial flora of the respective slides was maintained as nearly identical as possible by 'cross infection' of the various slides at the daily transfers. While no attempt was made to have the culture medium identical from day to day, the various lines whose division rates were to be compared were supplied with identical culture medium and were treated

exactly alike at the time of transfer. This, of course, was of critical importance. Furthermore, all isolations for the lines under comparison were made at the same time and with the same pipette, extreme care being taken to avoid mixing the individuals from different lines. The animals selected were isolated in equal quantities of the culture medium and kept under environmental conditions as nearly identical as it was possible to make them.

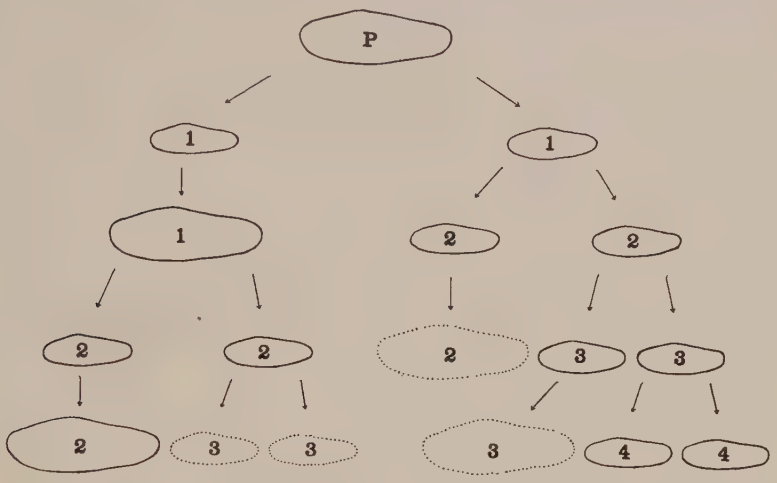


Fig. 1 Diagram of successive fissions among the progeny of a single individual, illustrating the variations in fission rate which formed the basis of selection in the present work. The left side of the figure traces a series of slow selections, the right side a series of fast selections, showing that at a given moment we may have, among the progeny of a single parent, individuals of the second and fourth generations. Animals discarded at isolation are indicated by broken lines.

Each depression slide contained ordinarily two parent individuals, the products of a single fission. The slides were examined daily or more often. When it was found that one of the animals on a slide had divided, while the other had not, a selection was made (fig. 1). If the particular line was being selected for a rapid rate of division, the two daughter cells of the animal which had divided were transferred to a new slide to become the parent animals for the next selection, while the undivided individual was discarded. Such selections

were designated as 'plus' selections. If, on the other hand, the line was being selected for a slow rate of division, the animal which had not divided was retained for the propagation of the race, while the other two were rejected. This constituted a 'minus' selection. In this manner a selection was made and recorded whenever animals belonging to different generations were observed among the progeny of a single parent or of two parents, themselves belonging to the same generation. Size differences between young and old animals afforded the necessary index for computing the ages of the various animals occurring on a given culture slide at the time of observation. Further details regarding the constitution of the lines will be presented in the descriptions of the various experiments.

The formulae brought together by Davenport ('99) and 'Student' ('08) have been employed in computing the constants and in determining the significance of the results obtained. All computations were checked by the aid of a Marchant mechanical calculator.

EXPERIMENTS AND RESULTS

1. Paramecium aurelia

Opposite selection (experiment A). This experiment extended over a period of 300 days from July 26, 1925, to May 21, 1926. From the progeny of a single animal two groups of eight lines each and a third group of four lines were isolated. Four lines from each of the first two groups were subjected to what is referred to as fast, or plus, selection over this period; the other four to slow, or minus, selection. These groups were designated as A and B, respectively. The four fast lines and the four slow lines comprising each group were accommodated in the same moist chamber. This rendered the temperature practically identical for the various lines under comparison. The third group of four lines was carried as a control set. These lines were treated as normal pedigree lines free from any definite selection process.

However, as already shown, there is rarely synchrony of fission in very closely related cells. This condition makes selection necessary, and, as such, it is practiced in every pedigree line of protozoa, consciously or unconsciously, as the case may be. Therefore, to eliminate the personal factor involved in such a process, 'balanced' selection (which amounts to no selection) was practiced in these lines. That is, each plus selection was recorded and followed by a minus selection at the next opportunity, and vice versa. In this way the effect of each selection was balanced by the one following.

Corresponding sets of stock lines were maintained daily throughout the experiment in order to provide animals for substitution whenever individuals of the main lines died or were otherwise lost through accident.

At the two hundred-day point two sets of sister animals were isolated from each of the main lines of A and B. These daughter lines furnished the material for the next two experiments described in the present paper. Unfortunately, however, one set of eight lines from the B series was lost through accident before the termination of the experiment. This made it necessary to discard the other set from the main series, since it could not be justly compared with the set from the A series which corresponded to the one lost.

Thus, while two main sets, each consisting of four fast-selected lines and four slow-selected lines, were carried for the full period of 300 days, the daughter lines from the second (B) set were lost to the experiment. Hence, to make for uniformity, all of the data presented in the tables and figures were derived from the A series, which, as stated above, consisted of four fast-selected lines and four slow-selected lines.

From time to time throughout the various experiments a greater number of lines were carried than it was possible to continue to the end. It was found that four sister lines gave, for all practical purposes, an average division rate almost identical with that for eight or more, and the smaller

number of lines allowed much greater care to be given to culture conditions and the selection process itself. To bear this out, averages showing the result of selection for the complete set of sixteen lines (A and B combined) will be presented as a separate item and may be compared with those from eight. It is believed that the clear and consistent results derived from these lines justify their consideration.

The data from experiment A is summarized in figures 2 and 3 and in tables 1 and 2, so that only a brief discussion

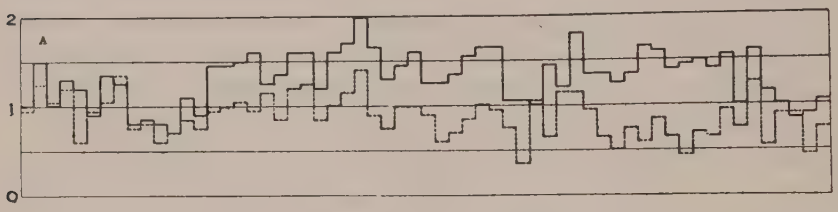


Fig. 2 Graph of the average number of generations produced, per line, by the fast and slow selected lines of Paramecium aurelia undergoing opposite selection; averaged for the sixty consecutive five-day periods of experiment A. The continuous line shows the average for the fast lines; the broken one, the average for the slow lines. The ordinates give the average number of generations produced and the abscissae, the number of days since the beginning of the experiment—the successive five-day periods.

is necessary in order to present the results of the 300 days of opposite selection.

Figure 2 is a graph which shows the average number of generations produced, per line, by the fast and slow selected sets of lines of experiment A for each successive five-day period; sixty five-day periods are plotted.

During this time the four plus selected lines underwent an average of 401 ± 3.11 divisions per line with a total of 341 selections, or 85.25 selections per line; the four minus selected lines, 275 ± 1.04 divisions per line with a total of 297 selections, or 74.25 per line. Thus, at the end of 300 days there was a very significant difference of 126 ± 3.27 generations between the fast and the slow lines. At this time

the fast lines were dividing at an average rate of 1.34 divisions per day and the slow lines at a rate of 0.92 divisions per day. The fast lines were dividing 45.6 per cent faster than the slow lines.

Table 1 gives the number of generations attained by the two diverse sets at the end of each fifty-day period for the first 200 days and (in the first column of the table) for each twenty-five-day period thereafter. Table 2 presents the same

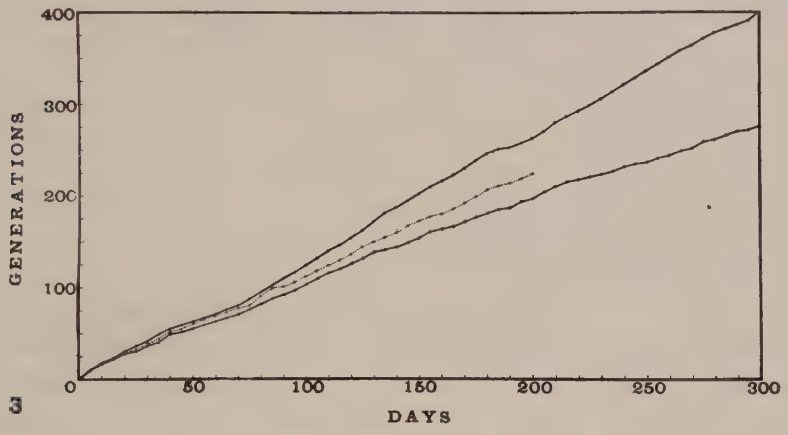


Fig. 3 Curves of the average number of generations produced, per line, by the fast and slow selected lines of Paramecium aurelia undergoing opposite selection, and the control lines with no selection; averaged and plotted at the end of each five-day period. The upper curve shows the average for the fast lines; the lower curve, the average for the slow lines, and the broken curve shows the average for the control. The ordinates give the average number of generations produced and the abscissae the number of days since the beginning of the experiment.

data, figured, however, on the basis of the number of generations per line per day with the percentage of excess in favor of the fast lines shown for each period.

Curves have been constructed (fig. 3) which show the fast and slow selected lines for the sixty five-day periods of the experiment and also the control lines for forty five-day periods, at the end of which time they were terminated. From the beginning of the experiment the control lines follow a course which is roughly midway between the two extremes

brought about by opposite selection. Also, the position of the control lines seems to indicate that plus selection gave the greater effect in bringing about the marked diversity obtained. Beyond one hundred days, the excess in division rate of the fast lines over the control lines is much greater than the excess of the control over the slow.

At the 200-day period, the mean number of divisions for the three sets—fast, slow, and control—were as follows:

Fast lines,	262.50 ± 0.77
Control lines,	223.25 ± 1.33
Slow lines,	196.50 ± 0.92

Here we have a distinct difference of 66.00 ± 1.20 generations between the fast lines and the slow lines. It is noted that the fast lines have an increase in division rate of 17.6 per cent over the control lines, while the control lines are in excess of the slow lines by 13.6 per cent.

The question now arises as to whether this difference is significant, whether the results justify the conclusion that we draw, namely, that selection has been effective in isolating diverse strains with reference to the physiological character, division rate. However, by digressing to present a mathematical demonstration of the significance of this difference, it will be possible to give for the same sample the correction for the limited number of lines upon which the study was based.

To gauge more accurately the significance of data resulting from a small number of cases, 'Student' ('08) has con structed tables which give the probability that the value of the mean (x), measured from the mean of the population, in terms of the standard deviation (s) of the sample, will lie between $-\infty$ and z, the distribution of z being represented by the quantity $\frac{x}{s}$. Thus, to take the table for samples of four ($n = 4$), the number of lines which we are considering, the probability that the mean of the population lies between $-\infty$ and once the standard deviation of the sample is 0.9092, or the odds are about ten to one that the mean of the population lies between these limits. When $n = 4$, the highest value

of z tabulated by 'Student' is that obtained when the mean is three times the standard deviation. In this case the probability of the mean of the population lying between $-\infty$ and three times the standard deviation of the sample is 0.9931, or the odds are about 143 to one that the mean of the population lies between these limits.

After two hundred days of opposite selection, the number of generations attained by the four fast and the four slow lines with the means and constants for each set, as well as for the differences, are as follows:

Line	*Fast*	*Slow*	*Difference (F-S)*
1	262	198	64
2	264	199	65
3	265	197	68
4	259	192	67
Mean	262.50	196.50	66.00
S.D.	± 2.29	± 2.74	± 1.58
P.E.	± 0.77	± 0.92	± 0.53

Therefore, $z = \frac{x}{s} = \frac{M}{S.D.} = \frac{66.00}{1.58} = 41.74$

If the mean difference between the number of generations attained by the fast and slow lines in 200 days were only three times the standard deviation of the four differences, then the probability that the true mean of the differences lies between the limits 64.42 and 67.58 would be 0.9862, and that it lies outside of these limits would be 0.0138. The chances therefore would be about seventy-one to one that the true mean lies within these limits. Hence, we may argue that, as 41.74 is greater than 3, so will the odds against the difference resulting from chance be infinitely greater than the probability ratio expressed when $z = 3$.

While these constants vary for each period directly with the distance of the individual lines from the mean of the group, the example just cited will suffice to illustrate the reliability of the differences which have been obtained. At the termination of the experiment, and after 300 days of selection, the difference is much greater and consequently infinitely more significant.

Averaging the second group of fast and slow lines with the first group, we have a total of eight fast-selected lines and eight slow-selected lines, as follows:

'F' lines (8): 394 ± 2.34 generations in 300 days, or 1.31 gens. per day.
'S' lines (8): 274 ± 1.48 generations in 300 days, or 0.91 gens. per day.
Difference: 120 ± 2.77 generations in 300 days, or 0.40 gens. per day.

It is seen that the results are essentially equivalent to those derived from four lines of each.

In a subsequent part of the paper an analysis will be made of the accumulation of this diversity and the rôle of selection in bringing it about. It is believed that some light will be brought to bear on the origin of these slight variations in division rate.

Balanced selection (*experiment* A_1). On February 11, 1926, a sister line was branched from each of the 'A' lines. At that time these lines had been undergoing continuous opposite selection for 200 days, and the average difference per line had increased from 7 ± 0.58 generations for the first fifty days to 66 ± 1.20 generations for the full period of 200 days (table 1). The fast lines were then dividing at the rate of 1.31 generations per line per day, while the division rate for the slow lines was 0.98 generation per line per day. This shows a marked excess of 33.7 per cent in favor of the fast lines (table 2). In order to test the permanence of this apparent effect of selection, the daughter lines were now subjected to 'balanced' selection, which amounts to no selection, for one hundred days. That is, each plus selection was followed by a minus selection at the next opportunity, and vice versa. As in the control lines of experiment A, the effect of each selection was balanced by the one following. The results of this experiment (A_1) are set forth in some detail in figure 4 and in tables 1, 2, and 3.

Figure 4 consists of three graphs, the first of which shows the division rate of the fast and slow selected A lines for the last one hundred days of the experiment, while the second gives the history of the non-selected 'balanced' lines (A_1) for the same period, averaged, as in figure 2, for consecutive

five-day periods. The third graph will be dealt with in connection with the next experiment.

At the termination of the one hundred days of balanced selection, the four non-selected fast lines, which had previously been subjected to 200 days of plus selection, had attained an average of 391 ± 2.08 divisions per line, and the

TABLE 1

A. Opposite selection (Paramecium aurelia)

50 days	'Fast' lines,	63 ± 0.29 generations per line
	'Slow' lines,	56 ± 0.50 generations per line
	Difference,	7 ± 0.58 generations per line
100 days	'Fast' lines,	124 ± 0.71 generations per line
	'Slow' lines,	103 ± 0.28 generations per line
	Difference,	21 ± 0.77 generations per line
150 days	'Fast' lines,	202 ± 1.95 generations per line
	'Slow' lines,	153 ± 1.31 generations per line
	Difference,	49 ± 1.86 generations per line
200 days	'Fast' lines,	262 ± 0.77 generations per line
	'Slow' lines,	196 ± 0.92 generations per line
	Difference,	66 ± 1.20 generations per line

A. OPPOSITE SELECTION		A_1. BALANCED SELECTION		A_2. REVERSED SELECTION	
225 days:		225 days:		225 days:	
'F' lines,	298 ± 1.66	'F' lines,	295 ± 0.92	'F' lines,	295 ± 0.90
'S' lines,	220 ± 0.55	'S' lines,	219 ± 0.69	'S' lines	223 ± 0.84
Difference,	78 ± 1.75	Difference,	76 ± 1.15	Difference,	72 ± 1.23
250 days:		250 days:		250 days:	
'F' lines,	336 ± 1.38	'F' lines,	326 ± 1.08	'F' lines,	327 ± 1.62
'S' lines,	236 ± 0.99	'S' lines,	237 ± 1.55	'S' lines,	244 ± 2.45
Difference,	100 ± 1.70	Difference,	89 ± 1.89	Difference,	83 ± 2.94
275 days:		275 days:		275 days:	
'F' lines,	371 ± 1.63	'F' lines,	361 ± 1.88	'F' lines,	348 ± 2.82
'S' lines,	258 ± 1.34	'S' lines,	261 ± 2.04	'S' lines,	267 ± 3.65
Difference,	113 ± 2.11	Difference,	100 ± 2.77	Difference,	81 ± 4.62
300 days:		300 days:		300 days:	
'F' lines,	401 ± 3.11	'F' lines,	391 ± 2.08	'F' lines,	375 ± 2.19
'S' lines,	275 ± 1.04	'S' lines,	272 ± 0.60	'S' lines,	286 ± 3.15
Difference,	126 ± 3.27	Difference,	119 ± 2.16	Difference,	89 ± 3.84

TABLE 2

A. Opposite selection (Paramecium aurelia)

50 days	'Fast' lines,	1.26 generations per line per day
	'Slow' lines,	1.12 generations per line per day
	Excess in favor of 'fast' lines, 12.5 per cent	
100 days	'Fast' lines,	1.24 generations per line per day
	'Slow' lines,	1.03 generations per line per day
	Excess in favor of 'fast' lines, 20.4 per cent	
150 days	'Fast' lines,	1.35 generations per line per day
	'Slow' lines,	1.02 generations per line per day
	Excess in favor of 'fast' lines, 32.3 per cent	
200 days	'Fast' lines,	1.31 generations per line per day
	'Slow' lines,	.98 generations per line per day
	Excess in favor of 'fast' lines, 33.7 per cent	

A. OPPOSITE SELECTION			A_1. BALANCED SELECTION			A_2. REVERSED SELECTION		
225 days:			225 days:			225 days:		
'F' lines,	1.32		'F' lines,	1.31		'F' lines,	1.31	
'S' lines,	.98		'S' lines,	.97		'S' lines,	.99	
Excess,		34.7%	Excess,		35.0%	Excess,		32.3%
250 days:			250 days:			250 days:		
'F' lines,	1.34		'F' lines,	1.30		'F' lines,	1.31	
'S' lines,	.94		'S' lines,	.95		'S' lines,	.98	
Excess,		42.5%	Excess,		36.8%	Excess,		33.7%
275 days:			275 days:			275 days:		
'F' lines,	1.35		'F' lines,	1.31		'F' lines,	1.26	
'S' lines,	.94		'S' lines,	.95		'S' lines,	.97	
Excess,		43.6%	Excess,		37.9%	Excess,		29.9%
300 days:			300 days:			300 days:		
'F' lines,	1.34		'F' lines,	1.30		'F' lines,	1.25	
'S' lines,	.92		'S' lines,	.91		'S' lines,	.95	
Excess,		45.6%	Excess,		42.8%	Excess,		31.6%

Fig. 4 Graphs showing the average number of generations per line per five-day period produced by the A lines, the A_1 lines and the A_2 lines, over the same period, 200 to 300 days. The A lines were subjected to continuous opposite selection for 300 days; the first graph shows the division rate for the last twenty consecutive five-day periods of their history. The second graph shows the average number of generations per line per five-day period produced by the non-selected 'fast' and 'slow' lines of experiment A_1 (balanced selection), previously subjected to opposite selection. The third graph shows the same for the slow selected 'fast' lines and the fast selected 'slow' lines of experiment A_2 (reversed selection). Method of plotting as in figure 2.

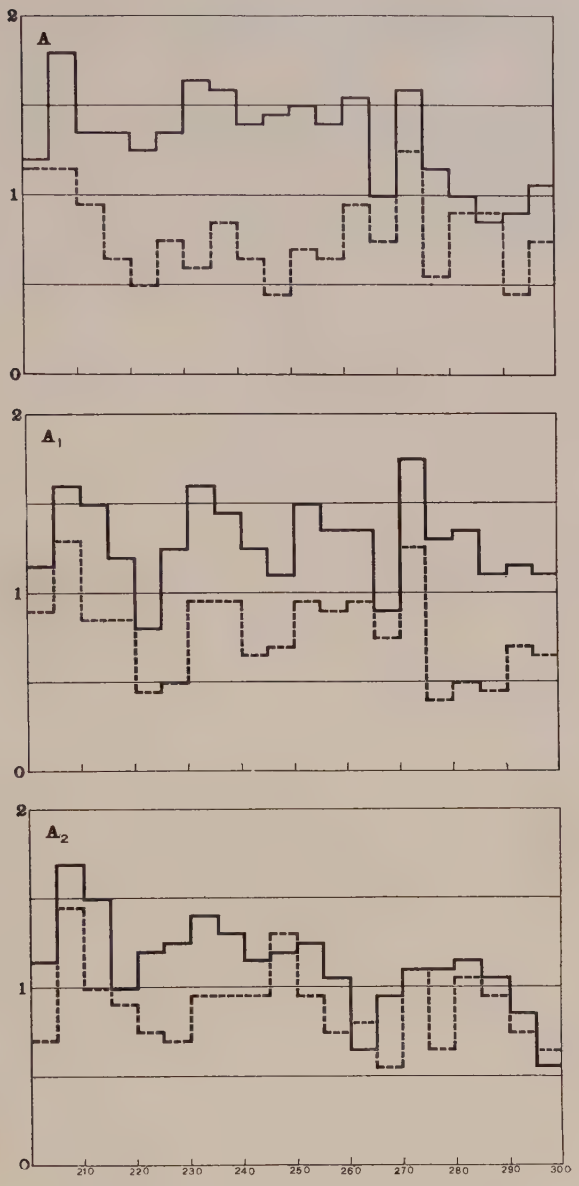


Figure 4

four non-selected slow lines, which had previously been treated to minus selection for 200 days, had undergone an average of 272 ± 0.60 generations per line over the same period of time. Thus, at the end of 300 days, there was a difference of 119 ± 2.16 generations between the 'fast' and 'slow' lines (table 1). At this time the 'fast' lines were dividing at an average rate of 1.30 divisions per day and the slow lines at a rate of 0.91 divisions per day. The excess was then 42.8 per cent in favor of the 'fast' lines (table 2).

TABLE 3

Paramecium aurelia

	Generations per line
A. Opposite selection, 300 days,	
'Fast' lines (200 to 300 days),	139
'Slow' lines (200 to 300 days),	79
Excess in favor of fast selected lines,	60
A_1. Opposite selection, 200 days,	
Balanced selection, 100 days,	
'Fast'—balanced lines (100 days),	129
'Slow'—balanced lines (100 days),	76
Excess in favor of lines previously fast selected,	53
A_2. Opposite selection, 200 days,	
Reversed selection, 100 days,	
'Fast'—reversed lines (100 days),	113
'Slow'—reversed lines (100 days),	90
Excess in favor of slow selected 'fast' lines,	23

Table 3 gives the number of generations produced by the 'fast' and 'slow' lines while subjected to the one hundred days of balanced selection. It will be noted that the 'fast' lines averaged 129 generations per line, while the 'slow' lines averaged but seventy-six generations per line. In spite of the fact that these lines were free from selection for one hundred days, we have at the end of that time an excess of fifty-three generations in favor of the lines previously fast selected. These results will be discussed more fully in a later section of the paper.

Reversed selection (*experiment* A_2). Along with the lines which were derived from the main 'A' lines on February 11,

1925, and subjected to balanced selection for one hundred days (experiment A), a second set was isolated at the same time and subjected to reversed selection for a similar period. That is, the lines which had been fast selected for 200 days were now minus selected, and the lines which had been slow selected for the same length of time were now plus selected. If the difference obtained by opposite selection was hereditary and the result of selection, then the same effect should be obtained a second time by reversing the process, so that the difference in fission rate between the 'fast' and 'slow' lines would be decreased. It was, of course, taken for granted that the effect produced by 200 days of opposite selection could not be entirely obliterated by reversed selection for a period only half as long, but it was hoped that such a process would give some indication, at least, of what had previously taken place. The results of this experiment are summarized in figure 4, and in tables 1, 2, and 3.

The third graph, in figure 4, shows the average number of generations produced, per line, by the minus selected 'fast' lines and the plus selected 'slow' lines for each successive five-day period; twenty five-day periods are plotted.

At the end of the one hundred days of reversed selection, the four minus selected 'fast' lines, which had previously been subjected to 200 days of plus selection, had attained an average of 375 ± 2.19 divisions per line, and the four plus selected 'slow' lines, which had been treated to minus selection for 200 days, had undergone an average of 286 ± 3.15 divisions per line over the same period of time. Thus, at the end of 300 days, there was a difference of 89 ± 3.84 generations between the 'fast' and 'slow' lines (table 1). During the one hundred days of reversed selection a total of ninety-two minus selections, or twenty-three per line, were made in the 'fast' lines, while the plus selections made in the 'slow' lines numbered 111, or 27.7 per line.

At the termination of reversed selection, the minus selected 'fast' lines were dividing at an average rate of 1.25 divisions per day and the plus selected 'slow' lines at a rate of 0.95

division per day. The excess was then 31.6 per cent in favor of the minus selected 'fast' lines.

It should be noted that the minus selected 'fast' lines produced an average of 113 generations per line during reversed selection, while the plus selected 'slow' averaged ninety generations per line (table 3).

In the next section of the paper an effort will be made to interpret these results in the light of those obtained in the previous experiment.

Comparison of results of experiments A, A_1, and A_2. Since the two sets of lines which were derived from the plus and minus selected lines at 200 days and which were subjected to balanced and reversed selection, respectively, were carried over the same period of time (one hundred days), it will be of considerable interest to compare the data obtained from each with that obtained by continued opposite selection in the main diverse lines. Figure 4 and tables 1, 2, and 3 have been so constructed that such a comparison can be readily made.

The balanced and reversed lines had a common history up to the 200-day point, i.e., they were both derived at that time from the plus and minus selected lines of experiment A. Also, opposite selection was continued in the main lines for one hundred days, during which time balanced and reversed selection were practiced in the sublines. The results of the three types of selection, which were performed on animals of like descent, are graphically presented in figure 4. A superficial glance will suffice to note what has actually taken place.

The main 'A' lines (opposite selection) not only maintained a marked diversity in division rate, but the difference already acquired was progressively increased by selection. As already indicated, that diversity increased until we have a difference of 126 ± 3.27 generations between the fast and the slow lines at the end of 300 days. This difference had increased by sixty generations in one hundred days (table 1).

Throughout this same period the lines subjected to balanced selection (A_1) maintained a difference in division rate of 119 ± 2.16 generations, while the difference in the case of the reversed lines (A_2) had dropped to 89 ± 3.84 generations per line. Or, expressed in somewhat different terms, at the end of 200 days of opposite selection the daily division rate of the plus-selected lines was 33.7 per cent faster than that of the minus-selected lines. Continued selection increased this difference to 45.6 per cent in 300 days. The 'fast' lines under balanced selection showed an excess almost as great, 42.8 per cent, while in the case of the minus selected 'fast' lines of the reversed set the excess had dropped to 31.6 per cent (table 2).

While it is surprising that the difference increased in the lines supposedly free from selection (A_1) to almost the same extent that it did in the selected lines (A), two possible explanations may be offered. In the first place, it is conceivable that a point was reached in plus selection, and before the termination of the experiment, beyond which selection was powerless to produce animals with a faster rate of division. It is also quite probable that, for a given race, there should be a minimum rate, below which death results. In fact, many of the substitutions recorded for the slow lines may have been necessitated by just such a condition. Such a state of affairs would prevent further divergence of the fast and slow lines after a certain point had been reached, and would allow the balanced lines to approach them—especially if the end-point came before 200 days. Another explanation of the progressive divergence in the balanced selected lines comparable to that in the lines under continued opposite selection might well be offered. Balanced selection, as already indicated, simply consisted in an alternation of plus and minus selection with an aim to balance the effect of the one by the next to follow. Now, if there should be certain periods in the life-cycle where selection may be effective and at all other times in vain, then some few selections made in the balanced process may have had just as great an effect in

producing diversity as others made at the appropriate time in the plus and minus selected lines of 'A.'

From the data presented in table 2 we note that, while the percentage of excess in favor of the fast lines had decreased after 100 days of reversed selection, at the same time it was not a gradual decline. This was due to the fact that the plus selected 'slow' lines showed a slight decrease in division rate rather than the expected increase. It should be observed, however, that this rate never dropped to the level that it did in the minus selected 'A' lines fifty days previously. Here again, while the total number of selections practiced may have been powerless to produce a great rise in the fission rate of the slow lines, at the same time a certain number may have been effective in preventing as marked a drop as was evidenced in the slow selected lines of experiment A. The next section of the paper will be devoted to a study of the periods of greatest effect.

Reversed selection apparently was effective in bringing the diverging lines somewhat closer together by essentially the same progressive increments as those by which they were pulled apart in the first 200 days of opposite selection. While in the lines under opposite selection the difference in favor of the fast lines increased by sixty generations and by fifty-three generations in the balanced selected lines, it increased only by twenty-three generations in the lines under reversed selection (table 3).

Periods of greatest effect from selection. If it is possible to obtain, through long-continued selection among the progeny of an animal which reproduces by fission, two sets of animals which differ characteristically in rate of fission, it follows that the divergence must either be correlated with the number of selections practiced throughout the whole period or else with appropriate selections made at susceptible periods in the life-history of the organism, if such can be shown to occur. The data have been analyzed with these two possibilities in mind, and the findings are presented in tables 4 and 5.

In order to test the first possibility, it will be necessary to examine the periods of greatest effect and to determine whether or not they coincide with the periods of greatest selection. In table 4 the average plus selections per line per ten-day period have been added to the average minus selections per line per ten-day period to give the total number of selections which were made in an effort to pull apart the two series of lines. This table also gives, for each ten-day period, the average number of generations per line per day in the fast and slow lines, with the percentage of excess in favor of the 'fast.'

Adding the average number of plus selections per line for the thirty ten-day periods to the average number of minus selections per line, we have a total of 159.5 selections for the full period of 300 days. This gives an average of 5.3 selections per ten-day period. An examination of the data given in figure 4 will show that in thirteen of the thirty ten-day periods the number of selections is above this average, and, furthermore, that in six of these the number ranges from 6.5 to 8.7. These six periods (4th, 14th, 15th, 18th, 20th, and 26th) will be arbitrarily spoken of as the periods of greatest selection.

We shall now examine the data in order to determine the periods of greatest effect. It is found that the percentage of excess of the fast lines over the slow lines per ten-day period ranges from 2.2 per cent to 158.2 per cent. The average excess of the fast lines over the slow lines for the thirty periods is 54.4 per cent for each ten-day period. As would be expected, the differences between the two sets of lines for the first ten periods are considerably lower than they are for the second and third groups of ten. For this reason the percentages have been averaged separately for the three groups of periods. They are as follows: periods 1 to 10, 24.2 per cent; periods 11 to 20, 62.0 per cent; periods 21 to 30, 76.9 per cent. Two periods showing the highest percentage of excess were selected from each group to give a total of six (8th, 9th, 17th, 19th, 24th, and 25th). These will be considered as the periods of greatest effect.

TABLE 4—Part 1

Paramecium aurelia

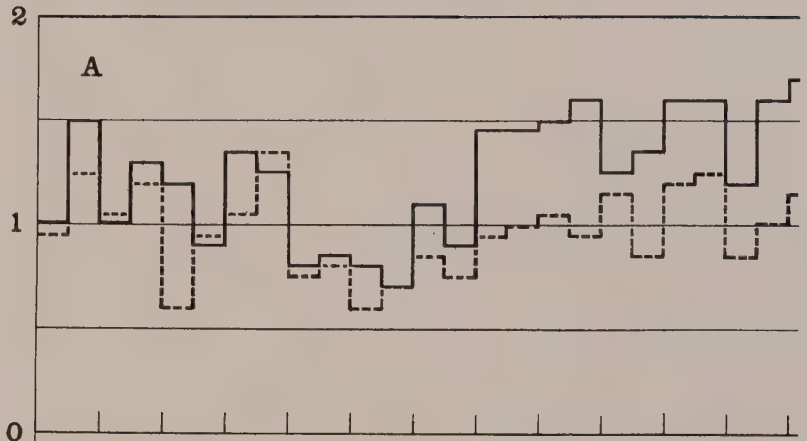
Opposite Selection

Polygon of average number of generations per line

60 five-day periods

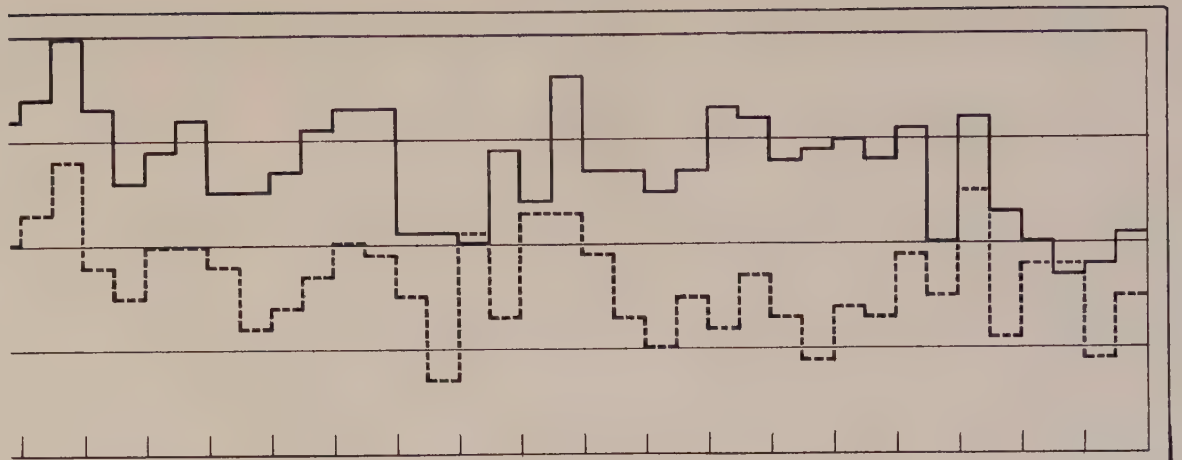
'Fast' lines

'Slow' lines



30 ten-day periods		1st.	2nd.	3rd.	4th.	5th.	6th.	7th.	8th.	9th.	10th.	11th.	12th.
Aver. '+' selections per line plus aver. '_' selections per line		2.2	3.2	5.0	6.5	4.7	3.7	4.5	4.7	4.7	5.0	6.2	6.0
Average number of generations per line per day	Fast:	1.25	1.15	1.05	1.30	.82	.75	1.00	1.45	1.55	1.30	1.60	1.40
	Slow:	1.10	1.12	.77	1.20	.77	.65	.80	.97	1.00	1.00	1.22	.92
Percent excess in favor of 'fast' lines		13.6%	2.7%	36.4%	8.3%	6.5%	15.4%	25.0%	49.5%	55.0%	30.0%	31.1%	52.2%

TABLE 4—Part 2



13th.	14th.	15th.	16th.	17th.	18th.	19th.	20th.	21st.	22nd.	23rd	24th.	25th.	26th.	27th.	28th.	29th.	30th.
6.0	7.7	8.7	4.5	4.5	7.0	6.0	7.0	6.0	5.5	4.2	5.2	5.2	6.5	5.0	6.0	4.0	4.0
1.85	1.47	1.52	1.25	1.45	1.65	1.05	1.22	1.50	1.35	1.30	1.62	1.42	1.45	1.27	1.37	.92	.97
1.27	.82	1.00	.75	.77	.97	.55	.85	1.15	.80	.62	.72	.55	.67	85	.90	.90	.62
45.7%	79.3%	52.0%	66.7%	88.3%	70.1%	90.9%	43.5%	30.4%	68.7%	109.7%	125.0%	158.2%	116.4%	49.4%	52.2%	2.2%	56.4%

Table 5 will aid in comparing the six periods of greatest selection with the six periods of greatest effect. It happens that the two series are made up of entirely different periods—no period is common to both. Also, of the six periods of greatest selection, only three show a percentage of excess higher than the average for the thirty periods, and of the six periods of greatest effect, only one shows more than the average number of selections. It is noted that 6.5 selections

TABLE 5

Periods of greatest selection

Ten-day period	*Selections*	*Per cent excess*
4th,	6.5	8.3
14th,	7.7	79.3
15th,	8.7	52.0
18th,	7.0	70.1
20th,	7.0	43.5
26th,	6.5	116.4

Periods of greatest effect

Periods 1 to 10; average excess in favor of 'fast,' 24.2 per cent.
8th ten-day period, 4.7 selections, 49.5 per cent of excess.
9th ten-day period, 4.7 selections, 55.0 per cent of excess.

Periods 11 to 20; average excess in favor of 'fast,' 62.0 per cent.
17th ten-day period, 4.5 selections, 83.3 per cent of excess.
19th ten-day period, 6.0 selections, 90.9 per cent of excess.

Periods 21 to 30; average excess in favor of 'fast,' 76.9 per cent.
24th ten-day period, 5.2 selections, 125.0 per cent of excess.
25th ten-day period, 5.2 selections, 158.2 per cent of excess.

may produce either an excess of 8.3 per cent or an excess of 116.4 per cent. In fact, it can be seen from the graph reproduced in table 4 that none of the periods of greatest selection are followed either by a striking increase in division rate of the fast or a marked decrease in the case of the slow. In some instances it so happens that it is quite the reverse. And where this is not true the rise or fall would be expected from the general nature of the rhythm at that particular point.

It might well be argued that, if the effect does not show itself in the immediate period of intensive selection, it may be demonstrated to occur in the period immediately following. An examination of table 4, however, will reveal the fact that, of the six periods immediately following the six periods of greatest selection, only two periods show a percentage of excess in favor of the fast lines, which is above the average for the thirty ten-day periods.

Hence, from a study of the evidence at hand, we conclude that, in Paramecium aurelia, the number of selections practiced, at any given time, in no way determines the effect thereby produced either at the time of selection itself or immediately thereafter.

It has already been suggested that the phenomenon of endomixis may have some bearing on the production of heritable variations in the Infusoria. As previously stated, Erdmann ('20) has found that, following endomixis in Paramecium aurelia, different lines with less or greater differences in size appear. It has already been shown that there is no correlation between the number of selections performed and the effect produced. Since it was clear from the results of experiment A that the diversity was solely due to selection, it follows that there must have been certain periods during the course of the experiment when some few selections were instrumental in bringing about this effect.

It is clearly indicated that the difference obtained was not accumulated gradually, but rather by irregular increments. To illustrate, the percentages of difference in proportion to the total average fission rate for each of the ten consecutive ten-day periods of opposite selection between 100 and 200 days are 9.4, 10.4, 11.0, 13.3, 13.8, 13.7, 14.5, 15.5, 15.0, and 14.4.

Woodruff and Erdmann ('14) and Woodruff ('17) showed that, in Paramecium aurelia, endomixis almost invariably occurs at the low point in the division rate between two rhythms, at intervals of about four weeks or about fifty generations, and that the synchronism of the process in different lines of the same race under identical conditions is remarkably exact.

If endomixis occurs at such points, it would be impossible to determine the degree to which the increased division rate, which marks the ascending phase, was influenced by selection. In the present experiment we note a very significant difference between the range of fluctuations in the division rate of the fast and slow lines. It is believed that this diversity was increased appreciably at certain of the endomictic periods where a few selections were made at what proved to be the appropriate time, and hence were effective. In the case of the fast lines, this would theoretically result in a steeper rhythm with the next reorganization process occurring before the division rate had dropped to a point as low as in the previous endomictic period. In the slow lines, on the other hand, the reverse would be true. After an effective selection the division rate would not increase to the extent that it had in the previous rhythm and would drop to a lower level before the next reorganization process set in. As can be seen from the graph in figure 2 or table 4, the first condition is actually the case in the fast lines for the four rhythms between the fifth and fourteenth ten-day periods, and again for the two between the nineteenth and twenty-fifth ten-day periods. And in the slow lines the reverse is characteristically true of the rhythms which occur between periods 9 and 19, and between periods 20 and 25. This, in the light of evidence previously presented, clearly suggests that heritable variations have originated at endomixis and have been sorted out by selection at that time.

It is quite probable that certain of the irregularities attending the accumulation of this diversity may have been due to the occurrence of endomixis at an unusual point in the rhythm. While the endomictic periods in general coincide with the periodic depressions in division rate, Woodruff and Erdmann have recorded rare instances in which the nuclear reorganization occurred at other points in the rhythm. Young ('18) has suggested that such irregularities occur quite frequently. Certain other irregularities may have resulted from a failure to make a selection at the time of the particular

micronuclear division during which variations may originate. Another opportunity would not present itself for twenty-five to thirty days. In both cases the result would be a deviation from the expected trend of the two sets of lines, or of one alone, which would make comparison of the two equally difficult.

In conclusion, it would seem that we are reasonably justified in assuming that, in Paramecium aurelia, the effectiveness of selection in breaking up a clone into two hereditarily diverse lines with respect to division rate depends upon appropriate selections performed at certain time intervals rather than upon a greater number performed at random. Furthermore, the evidence seems to indicate that endomixis has afforded the susceptible periods in the life-history at which time the characters of the race may be altered to a greater or less degree by selection. A subsequent portion of the paper will be devoted to further discussion of the rôle of endomixis in the effectiveness of selection.

2. Paramecium calkinsi

Opposite selection. The experiment with Paramecium calkinsi extended over a period of 200 days, from August 15, 1926, to March 3, 1927. From the progeny of a single animal five groups of eight lines each were isolated. Four lines from each of the first four groups were subjected to fast, or plus, selection over this period, the other four to slow, or minus, selection. These groups were designated as cultures I, II, III, and IV, respectively. As in the previous experiments, the four fast selected lines and the four slow selected lines comprising each group were accommodated in the same moist chamber. The fifth group, designated as culture V, was carried as a control set. These lines were carried as normal pedigree lines under balanced selection. As before, a complete set of stock lines was carried from the main selected and non-selected lines.

At the fifty-day point a series of major selections were made in the fast and slow lines of the first four cultures

(I to IV). That is, of the four fast selected lines in each culture, the line which had produced the greatest number of generations was retained, while the other three were discarded and replaced by sublines from the remaining, or fastest, line. Similarly, major selections were practiced in the slow selected lines of the four cultures by retaining the slowest line and discarding the others, substituting as before. Throughout the remaining 150 days of the experiment, opposite selection was continued without recourse to major selections. In the sixteen fast selected lines a total of 785 plus selections were performed, while a total of 834 minus selections were made in the sixteen slow selected lines. This gave an average of 49 plus selections per line and 52.1 minus selections per line, respectively.

TABLE 6

Paramecium calkinsi. Opposite selection—200 days

		Generations per line	*Generations per line per day*
Culture I,	'Fast' lines (4)	156	.78
	'Slow' lines (4)	160	.80
Culture II,	'Fast' lines (4)	168	.84
	'Slow' lines (4)	161	.80
Culture III,	'Fast' lines (4)	157	.78
	'Slow' lines (4)	149	.74
Culture IV,	'Fast' lines (4)	154	.77
	'Slow' lines (4)	145	.72
Culture V,	Control lines (8)	155	.77
	Summary		
Cultures I–IV,	'Fast' lines (16)	159	.79
Culture V,	Control lines (8)	155	.77
Cultures I–IV,	'Slow' lines (16)	154	.77

A summary of the results obtained is presented in table 6. During the period of 200 days, the sixteen fast selected lines underwent an average of 159 generations per line, or 0.79 generations per line per day, while the sixteen slow selected

lines produced 154 generations per line, or 0.77 generation per line per day. Over the same time the eight control lines underwent 155 generations per line, or 0.77 generation per line per day.

It is seen at once that, in spite of 200 days of opposite selection, it was not possible to produce two lines with an appreciable difference in division rate. In fact, the slow

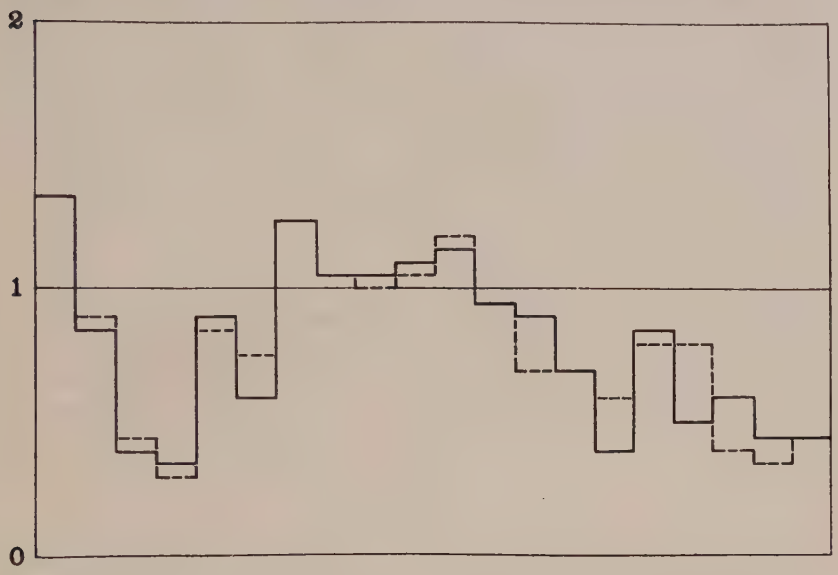


Fig. 5 Graph of the average number of generations produced, per line, by the fast and slow selected lines of Paramecium calkinsi, culture I, undergoing opposite selection; averaged for the twenty consecutive five-day periods between 100 and 200 days of selection. Method of plotting as in figure 2.

selected lines of culture I produced an average of 0.02 generation per day more than the fast selected lines. Also, in cultures I and II, the slow lines produced more generations than the control lines of culture V. However, these differences are all within the limits of error. It is sufficient to state that, in Paramecium calkinsi, selection was quite without effect in producing a diversity in division rate.

Figure 5 is a graph which shows the average division rate of the fast and slow selected lines of culture I for the last

one hundred days of the experiment, again averaged for five-day periods. Due to some irregularity in the synchrony of the rhythms of the four cultures under selection, it was felt that the results could be more accurately presented by a graph of one culture alone rather than by a graph combining the four. We note that there is no consistent excess in favor of either the fast or slow selected lines.

After a very careful study, Spencer ('24) showed that endomixis does not occur at any point in the life-history of Paramecium calkinsi. But, although endomixis is absent, "fluctuations in the rate of reproduction are present which apparently are indistinguishable from the rhythms of P. aurelia and P. caudatum." The graph of figure 5 shows these fluctuations which are not unlike those which occurred in Paramecium aurelia (fig. 2).

In our work with Paramecium aurelia we found evidence indicating that selection was the instrumental factor in establishing two lines which differed with respect to the physiological character, division rate. The production of this diversity was correlated with the occurrence of endomixis which afforded the susceptible periods when selection was effective. This conclusion seems to be further substantiated by the results of the present experiment. Endomixis is not present in Paramecium calkinsi, and hence there has been no opportunity for the production of heritable variations in this race through selection.

3. Stylonychia pustulata

Opposite selection. The experiment with Stylonychia pustulata was started on August 18, 1926. Sixteen fast selected lines, sixteen slow selected lines, and eight control lines were started from a single animal in the same manner as already described for Paramecium calkinsi. When the lines had been carried for a little over one hundred days, the experiment was unavoidably terminated by the death of several of the component lines.

The data obtained from the various fast, slow, and non-selected lines, for one hundred days (August 18th to November 26th), are presented in table 7. Also, figure 6 shows the average division rate of culture I again averaged for five-day periods.

In the sixteen fast selected lines an average of 38.2 plus selections per line were performed, while an average of 36.8 minus selections per line were made in the sixteen slow

TABLE 7

Stylonychia pustulata. Opposite selection—100 days

		Selections per line	*Generations per line*
Culture I,	'Fast' lines (4)	40.5	223
	'Slow' lines (4)	32.5	163
Culture II,	'Fast' lines (4)	36.5	216
	'Slow' lines (4)	35.0	180
Culture III,	'Fast' lines (4)	40.7	261
	'Slow' lines (4)	40.2	228
Culture IV,	'Fast' lines (4)	35.2	244
	'Slow' lines (4)	39.5	173
Culture V,	Control lines (8)		217
	Summary		
Cultures I–IV,	'Fast' lines (16)	38.2	236
Culture V,	Control lines (8)		217
Cultures I–IV,	'Slow' lines (16)	36.8	186

selected lines. At the fifty-day point, major selections were made in the fast and slow lines of the first four cultures.

During the period of one hundred days, the fast selected lines underwent an average of 236 generations per line (2.36 generations per line per day), while the slow selected lines produced 186 generations per line (1.86 generations per line per day). Over the same time, the control lines underwent 217 generations per line (2.17 generations per line per day). Hence, in one hundred days, we have a difference of fifty generations in favor of the fast selected lines, which is obviously a well-marked divergence.

These results compare very favorably with those obtained by Middleton ('15), working with the same species. As a result of 130 days of opposite selection, this author was able to obtain a difference between the two diverse sets which was "the equivalent of some 74 selections extending through

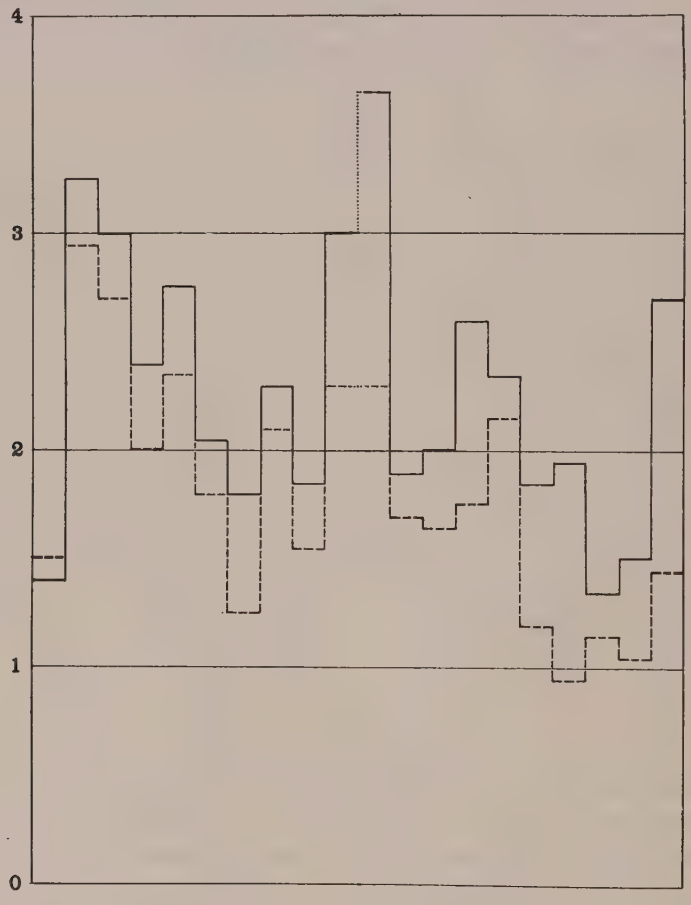


Fig. 6 Graph of the average number of generations produced, per line, by the fast and slow selected lines of Stylonychia pustulata, culture I, undergoing opposite selection; averaged for the twenty consecutive five-day periods of the experiment. Method of plotting as in figure 2. The dotted lines between the tenth and eleventh five-day periods indicate the performance of major selections at that point.

an average of 150 generations. This resulted in the production of a constant average difference per line of 0.42 of one fission per day.'' In the present experiment, which extended over a period of one hundred days, the equivalent of some seventy-five selections was performed, through an average of 211 generations. This resulted in the production of an average difference per line of 0.5 of one division per day.

The phenomenon of endomixis has never been observed during vegetative reproduction in Stylonychia pustulata. Fermor ('13) described an internal reorganization process during encystment in this species, which, however, involves nuclear fusions and is, therefore, not endomixis, but autogamy. However, Klee ('25) believes that she has discovered a reorganization process which occurs during vegetative reproduction in another hypotrich, Euplotes longipes. This process is held to be a form of endomixis midway between the type which Calkins ('19) found in Uroleptus mobilis during encystment and the 'Paramecium-type' described by Woodruff and Erdmann ('14) in free-living animals.

Since it is known that a process occurs in Stylonychia pustulata during encystment which involves a reorganization of nuclear elements (autogamy), and that a type of endomixis has been described in the free-living Euplotes longipes, it is entirely conceivable that such a process may also occur in Stylonychia during vegetative reproduction. Erdmann ('20), in a discussion of Middleton's experiments, suggests that, pending a reinvestigation of Fermor's discovery of 'autogamy' in Stylonychia pustulata, it is permissible to believe that a reorganization process occurs during encystment which is similar to endomixis. And granting that endomixis is excluded from these experiments, she holds that the effectiveness of selection in this species must be accounted for either by recognizable cytological variations or by a rearrangement of the germinal material which is without recognizable cytological signs. This seems to be a very logical conclusion, though without a factual basis. It seems to be even

more plausible when considered in the light of the results obtained in the present work on P. aurelia and P. calkinsi.

CONCLUSIONS

The facts gained from the experiments herein recorded indicate that the effectiveness of selection in producing divergent races depends, in some forms at least, upon the occurrence of a nuclear reorganization process in the life-history of the race. In Paramecium aurelia such a process is present in the internal regulatory phenomenon, endomixis. Woodruff and Erdmann ('14), in their description of this phenomenon, stated that "Endomixis, involving as it does the disintegration and absorption of the old macronuclear and micronuclear material, affords the opportunity for molecular rearrangement and therefore may afford the opportunity for the origin of variations within a pure line of Paramecium."

This is seen to be true for Paramecium aurelia—a form in which endomixis occurs. In Paramecium calkinsi, in which the process has never been demonstrated, and apparently does not occur, selection is quite without effect in producing diversity in rate of division. In Stylonychia pustulata, while selection is effective in establishing two diverse sets with regard to division rate, there is no direct evidence of the occurrence of a nuclear reorganization process in vegetative reproduction. However, as has been already pointed out, Erdmann ('20) suggests that even here endomixis may be found to be the causative factor involved.

Hence, from the evidence obtained from the selection experiments on Paramecium aurelia, Paramecium calkinsi, and Stylonychia pustulata, and from a consideration of the results of previous investigators, it is clear that different species do not lend themselves equally to the production of diverse strains by selection. To what constitutional factor or factors this difference is due has not been completely demonstrated. However, in those species which afford an opportunity for the effectiveness of selection such perform-

ances apparently must be made chiefly at certain 'sensitive' periods, e.g., endomixis, in order to produce a permanent change in the genotype. Accordingly, from the data at hand, it seems reasonable to conclude that nuclear reorganization processes afford conditions for selection to be effective.

SUMMARY

1. In Paramecium aurelia, by opposite selection, two diverse sets were established among the descendants of a single animal by vegetative reproduction, which differed in regard to the physiological character—division rate.

2. Control lines (non-selected), derived from the same animal in the beginning, followed a course roughly midway between the two extremes brought about by selection.

3. The difference obtained by opposite selection persisted through a period of balanced selection, which amounts to no selection.

4. Under the influence of reversed selection, following opposite selection, the diverging lines were brought somewhat closer together by essentially the same progressive increments as those by which they had been pulled apart during opposite selection.

5. From a comparative study of the periods of greatest selection and the periods of greatest effect, it was found that there was no relationship between the number of selections practiced at any given time and the effect produced.

6. The evidence from Paramecium aurelia seems to indicate that the effectiveness of selection in breaking up a clone into two hereditarily diverse lines with respect to division rate depends upon appropriate selections performed at certain time intervals (endomictic periods) rather than upon a greater number performed at random.

7. In Paramecium calkinsi, in which endomixis has never been observed, and apparently does not occur, selection was quite without effect in producing diversity.

8. In Stylonychia pustulata a well-marked divergence was obtained by selection, as has already been shown by Middle-

ton. It is probable that the effectiveness of selection in this species may be ascribed to a nuclear reorganization process, visible or otherwise, which involves a rearrangement of the germinal material.

9. Accordingly, from the data at hand, it seems reasonable to conclude that, in the Infusoria, certain nuclear reorganization processes afford conditions for selection to be effective.

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3779

Biometric Studies Upon Development and Growth in Amblystoma Punctatum and Tigrinum.

E. M. PATCH. (Introduced by J. S. Nicholas.)

From the Osborn Zoological Laboratory, Yale University.

Measurements of *Amblystoma punctatum* and *tigrinum* have been made in order to secure data upon the normal rate of growth and upon the modifications of this growth rate by experimental procedure. Data show that the rate of enlargement during embryonic stages is dependent upon the temperature, the size at any named stage being correlated with the size of the egg. Length increase in the embryo is made at the expense of the other dimensions. The curve of absolute increments of length when plotted against time during embryonic development is S-shaped, terminating at the end of this period.

The mean lengths of *Amblystoma punctatum, tigrinum,* and *Axolotls* at this point of development are 15.61 mm., 14.07 mm., and 11.96 mm., respectively. Tables for mean length, standard deviation coefficient of variation, and the probable errors of these quantities demonstrate the value of the criterion used.

Growth following the embryonic period is dependent upon food—quality, quantity, and frequency of feeding. Where feeding is alike, size relations of the embryonic period hold for larval growth. No food has been found as adaptable for early use as the natural diet which consists of small aquatic forms. Beef-liver can be used at early stages and produces a remarkable acceleration of growth. Of the tissues tried, kidney is second in value.

In the later larval development and adult life, beef muscle produces greater growth than liver; at these stages, liver feeding is attended by excessive glandular production, by lack of growth, and often with loss of appetite. Death sometimes follows. There is a weakening of normal peristaltic action, which can be compensated for, partly at

least, by use of agar. Beef muscle is of poor value for early larval growth and development. Here, as later, there is a lack of pigmentation with this food. In later larval and adult life, while the growth made on muscle is large, the mineral deficiency is evident—especially the calcium lack as shown by the tetanic condition.

Feeding on earthworms, with cuticle broken and digestive contents removed, produced little growth; the animals so fed are, however, apparently normal. *Enchytraei* (white worms) as food give a good growth rate; but the animals so fed, though large and well-formed, do not metamorphose. Animals fed on beef muscle will not metamorphose without addition of vitamins or minerals to their diet. Metamorphosis occurs successfully with liver and with earthworms, as is true with a mixed diet of the meats and worms. The latter, started in later larval life, produces the best growth of all diets. All of the reactions to foods are modified by the quality, quantity, frequency, and duration of the initial diet.

Use of synthetic diets emphasized the need for minerals and vitamins, the greater growth with vitamin A, showed greater growth with deptrin present, the value of low fat content, the preference for beef muscle powder as protein basis rather than the powdered liver, egg-white, egg-yolk, casein, or klim—though growth was made with all of these. The diets were more useful with older, larger larvae. Younger larvae showed different relative reactions.

During larval life the size relations of *Amblystoma punctatum* and *tigrinum* were reversed, so that at the time for metamorphosis the ratio was approximately 1 :2 in the order named. Larval life gave another S-cycle to the growth curve, this cycle terminating at the time of metamorphosis. A third life cycle, beginning at this point, is probably related to the development of sexual maturity. Plotting of the cube of length against weight, for measurements made on adults, shows the first quantity to be a linear function of the second.

Hibernation of adults was followed, on return to warm temperature and food, by a remarkable acceleration of growth until the size characteristic for the stage of development was reached or surpassed. The tail became proportionally shorter during the hibernation period, with return to normal proportion after renewal of growth. The index of build; weight/length, (Bardeen *et al*), was slowly decreased with increase of size.

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THE DETERMINATION OF THE AMOUNT OF HEMOGLOBIN PRESENT IN RAT FETUSES DURING DEVELOPMENT

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Received for publication October 22, 1927

It was found necessary during the author's experiments upon rat fetuses to determine the rate of hemoglobin development in this form (1), (2). The results secured by Williamson and Ets (3) show that the amount of hemoglobin falls perceptibly between the twentieth and the sixtieth days of post-natal development. They further indicate that the amount of hemoglobin present at birth is probably higher than their ten-day figures would indicate. Our own results place the amount of hemoglobin at birth slightly lower than the figure given by them. It is to be admitted, however, that our results must of necessity be more of an approximation than an absolute quantitative measure of the amount of hemoglobin present. This is due to the necessity of working with exceedingly small amounts of blood and also to the comparatively few cases which we were able to use. The results of further determinations may change the quantitative but not the comparative value.

The difficulty in obtaining results which are at all uniform when dealing with materials which are so highly variable in size relationships as embryos, will at once be appreciated by anyone who has had to use similar material.

It should be noted that the mean is more than 72 times the probable error. The probable error can be shown to be much less than the assigned value if it be calculated on the basis of a weighted mean. If the materials and quantities be considered it is rather remarkable that the error is so small.

The obtaining of blood from rat fetuses in sufficient quantity for hemoglobin estimation was the first problem to be presented. Pregnant females, selected and grouped according to the age of the fetuses as determined by palpation, were operated upon under light ether anesthesia. The horns of the uterus were slit in such a way as not to injure the placenta. The fetus was removed with the fetal membranes intact and the placenta attached. Immediately upon removal from the uterus, the fetuses were placed in warm physiological saline until the determination sample could be taken. The amnion was removed just before the fetus

was taken from the saline, total length and crown-rump measurements were made which were checked against the Wistar tables in determining age groupings (4).

The fetuses which were sixteen days or above in the gestation period present no difficulty in obtaining the necessary amount of blood. In animals younger than this, however, there is such a small available blood content that it was difficult to secure even the few drops necessary for a determination. Under the conditions of removal and subsequent keeping for a short period in saline, the fetal blood apparently flows toward the placenta. It was only after the placenta accidently was lifted above the plane of the embryo that the blood gravitated toward the fetus and could be collected from the neck vessels. In all cases the blood for determinations was secured by decapitating the animal and securing the blood from

TABLE 1

AGE GESTATION	NUMBER OF CASES	DETERMINATIONS	HEMOGLOBIN*	VALUE OF MEAN
days			*per cent*	*grams per 100 cc.*
12–13	11	55	30	5.12 ±0.025
14	14	70	32	5.45 ±0.083
15	16	80	37	6.34 ±0.071
16	7	35	55	9.36 ±0.025
17	22	110	56	9.40 ±0.057
18	10	50	45	7.69 ±0.029
20	6	30	44	7.52 ±0.074
21	20	100	54	9.22 ±0.070
Newborn	7	35	65	11.05 ±0.227
Totals.....	102	560	Mean for series	7.9314±0.11

* 16.92 grams = 100 per cent.

the vessels of the neck. While a slight amount of other body fluids probably is collected by this method, it is improbable that this is of sufficient quantity to vitiate the comparative result.

The determination of hemoglobin was carried out according to the Newcomer method and the percentages are based on Williamson's normal value for the human (16.92 grams per 100 cc. = 100 per cent). Five determinations were made of each sample and these were averaged before adding the total number of individual cases which were also averaged. The diluting fluid was 1 per cent hydrochloric acid, the dilution being 1:502, this solution being corrected for the elapsed time between the addition of the hydrochloric acid and the hemoglobin determination. Since the standard Bausch and Lomb colorimeters are not adapted to measuring some of the low hemoglobin contents with which we had to deal, it was

necessary for us to use the 20 mm. microcolorimeter of the Duboscq type. This instrument was standardised against readings of the same solutions made with the Newcomer glass filter standard against known solutions in the 40 mm. standard colorimeter. Our method of standardisation showed that the Newcomer method can be applied to the microcolorimeter with the readings running as low as 20 to 25 per cent hemoglobin.

Traces of hemoglobin could undoubtedly be found much earlier than the lowest figure given in the above table. To ensure a qualitative test particularly at early stages would be possible particularly with microhistological methods but unfortunately these would give no idea of the quantity present.

Our results show that hemoglobin is present in a measurable amount before the 14th day of the gestation period in the rat. That is to say, the development of hemoglobin in measurable amounts begins during the later part of the second third of gestation. From this point, there is a significant rise from the 14th day to the 17th day, the most abrupt portion of the curve coming in the period between the 15th to the 16th days. There is a fall in the amount of hemoglobin on the 18th day of the gestation period with a succeeding rise on the final two days of gestation. The newborn rats show a slight increase in hemoglobin quantity over the fetus of only a few hours before. This increase may or may not be significant, the number of cases being too small to say definitely.

SUMMARY

1. The amount of hemoglobin present in rat fetuses has been measured at different periods of development.

2. The quantity of hemoglobin shows a gradual increase during the later part of the second third of the gestation period.

3. An extremely rapid rise occurs in hemoglobin content during the early part of the third period of gestation. This is followed by a slight drop and by a recovery which shows an increasing amount of hemoglobin present shortly after birth.

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3819

Heteroplastic Transplantation of Halves of Girdle Rudiment in Amblystoma.

JOSEPH L. SCHWIND. (Introduced by J. S. Nicholas.)

From the Osborn Zoological Laboratory, Yale University.

In heteroplastic transplantations of the eye, Harrison[1] found that when the lens came from the donor species and the retina from the host species, each part exerted an influence on the other's growth. In order to see if a mosaic system would react in a similar way when the rudiment contained tissue from the two species, halves of the girdle rudiment were transplanted between *Amblystoma punctatum* and *A. tigrinum*. Reciprocal transplants of dorsal, ventral, anterior and posterior halves of the girdle rudiment were made. Except in a few cases killed for the study of intermediate stages, all animals were raised until the *tigrinum* of the pair had a fully formed fourth digit, usually 70 to 80 days after operation.

The limbs and girdles, both normal and operated, were prepared by a modified van Wihje method for the staining of cartilage, and after the muscles had been carefully removed, were mounted in damar. It was found that at 80 days there are well marked differences between the normal girdles of the two species. The *punctatum* girdle is smaller, has undergone considerably more chrondrification than that of the *tigrinum*, has a rounded coracoid and a short, stout scapula which passes gradually into the broader suprascapula. The *tigrinum* girdle is larger, having its coracoid shaped more like a plow, and having a very long, slender scapula at the top of which is a much broader suprascapula.

The specificity of the transplanted tissue was shown by the fact that there was no regulation of form due to transplantation into the girdle region of the host species. The portion of the operated girdle that had come from the donor could easily be recognized. The procoracoid and the muscles attached to the operated girdles were so variable that they could not be studied.

In the dorsal half transplantations, which included the rudiments of the free limb, scapula and suprascapula, these parts were always of the donor species. Under optimum conditions, the transplanted

limbs grew to the same size upon the host species as their control limbs did upon the donor species. The growth in size of the limbs is due to factors intrinsic to the graft. Cell counts showed hypoplasia of the spinal ganglia supplying the grafted limb in the case of a *tigrinum* bearing a *punctatum* limb. In the reverse combination, a *punctatum* bearing a *tigrinum* limb, there was hyperplasia of the ganglion cells. The effect upon the ganglia of *A. punctatum* was relatively greater than upon those of *A. tigrinum,* as was expected from the size of the limbs.

In the ventral half transplantations, which include the rudiments of the coracoid and procoracoid, the free limb, scapula and suprascapula are always of the host species. This is the only series in which complicating factors are not introduced by the presence of either a larger or smaller limb than normally would be present. It shows that the transplanted tissue from the donor species has no effect upon that part of the girdle supplied by the host species, for the scapula and suprascapula in all cases are of a size normal to the host. This series indicates that the tissue which has the potentialities of forming girdle material is more extensive than has hitherto been supposed.

In the transplantations of the anterior and posterior halves, the dividing line being taken as the projection of the fourth-fifth somite boundary, many of the limbs are compound; tissue from both species is present in the limb at the same time and capable of morphological identification. This is especially striking in the digits and carpus. In single limbs which contain tissue from both species, if the grafted tissue formed the anterior half, the anterior digits are of the donor species and the posterior digits are of the host species. If the posterior half of the rudiment was transplanted, the posterior digits are of the donor species and the anterior ones are of the host species. This shows that the tissue which is determined to form the digits is localized in the limb disc in an antero-posterior direction, the material for the first digit lying most anteriorly. With a few exceptions, the carpal elements are similarly affected. That is, a hand having the first two digits composed of *tigrinum* tissue will have carpales 1 and 2 and the radiale enlarged, while a hand having the first two digits composed of *punctatum* tissue will have carpales 1 and 2 and the radiale reduced.

The girdles of the posterior half series were very little or not at all affected by the operation, making it probable that there is little or no girdle-forming material posterior to the fourth-fifth somite boundary.

[1] Harrison, Ross G., *Anat. Rec.*, 1925, xxxi, 299.

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3921

Effect of Prenatal and Postnatal Injections of the Pituitary Gland in the White Rat.

E. L. COREY. (Introduced by J. S. Nicholas.)

From the Osborn Zoological Laboratory, Yale University.

The present study was undertaken in an attempt to ascertain the time at which the pituitary hormone begins to act upon the organism. Smith and Engle[1] have shown that transplantation of portions of the pars anterior of the pituitary into the young rat causes marked responses in the genital system attributable to the influence of the grafts upon the gonad, which in turn modifies the entire genital tract by means of its enhanced secretions. The gonad has, therefore, been selected as an organ in which there is a definite response to the pituitary hormone which might be used as an index of the first activity of the physiological effect of that secretion.

For the technique employed in performing laparotomy upon the pregnant rat preparatory to the injection of foetuses see Nicholas.[2] Previous failures of many workers to obtain positive results with injections of the gland, single transplants, and pituitary feeding renders the success of the method employed of some interest. The entire pituitary gland was excised from a freshly killed male or female adult rat and macerated under aseptic conditions in a ground-glass mortar in 1 cc. of 0.9% sodium chloride solution. The resultant fluid was used for injection, the same concentration as above being constantly maintained. In all prenatal injections a 27 gauge needle was used, and from .02 to .05 cc. were administered intraperitoneally at each injection. In postnatal treatment from 0.1 to 0.25 cc. were given, either intraperitoneally or subcutaneously, the type of injection being noted in the protocol. Prenatal injections were made upon alternate days; after birth, daily.

The youngest animals injected were those in the fifteenth day of

INJECTED ANIMALS.

Age (days)	No. litters	No. individuals
15 (Prenatal)	5	26
16 (Prenatal)	2	13
17 (Prenatal)	2	16
18 (Prenatal)	3	19
New-born	2	26

Four litters supplied the control animals.

foetal life. Sixteen, 17, and 18-day embryos were similarly injected. Gonads of both sexes were preserved for sectioning at various stages of both injected and non-injected animals. All material was fixed in Bouin's solution.

Prenatal injection in no way hastened the differentiation in the gonad. It is thus evident that the gonad of the rat foetus is incapable of response to the pituitary hormone. No evidence of sexual maturity was observed in either sex until the tenth day of postnatal life, when the effect was most pronounced in the male. This effect evinced itself in an apparent increase in tubule length as well as by a considerable thickening of the tubular wall. There is an undoubted increase in the size of the interstitial cells, and the tissue presents a dense and closely-packed appearance. We attribute this close-packed appearance to the increase in length and diameter of the tubules. No reconstructions have as yet been made to render this point a certainty.

In the ovary the earliest advance in maturity of the injected animals over the controls appeared at approximately the fifteenth day. No corpora lutea were present but the follicles are larger in diameter and more nearly approaching maturity than in the normal animal if judged by the amount of follicular fluid present. At the twentieth day this precocious development is more marked. In no case were corpora lutea observed nor could eggs be discerned within the lumen of the sectioned tube.

On the basis of the above results, the physiological effect of the pituitary hormone upon the gonad first shows itself in *both* sexes between the tenth and fifteenth days of postnatal life.

[1] Smith, P. E., and Engle, E. T., *Am. J. Anat.*, 1927, xl, 159.

[2] Nicholas, J. S., *Anat. Rec.*, 1925, xxxi, 385.

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EXPERIMENTAL STUDIES ON THE CILIARY ACTION OF AMPHIBIAN EMBRYOS

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TWELVE FIGURES

The presence of active cilia on the ectoderm of amphibian embryos is familiarly manifested by the rotation of the embryos within the egg capsules. The behavior of these cilia under normal conditions has been described by Assheton ('96) and by Woerdeman ('25); by the former in Rana temporaria and Triton cristatus, by the latter in Rana esculenta and the black axolotl. First appearing about the time the neural folds close, the cilia have their action directed caudally on most of the embryo, creating a corresponding current in the water, or propelling the embryo anteriorly along the dish when removed from its capsule.

The present study,[1] concerned primarily with certain responses of the ciliary action to experimental situations, was made chiefly on embryos of Amblystoma punctatum. After a brief account of the normal activity in this form, the experimental results will be treated under the following headings: determination of polarity of ciliated cells; reversal of ciliary action; effect of curare on ciliary action; and coordination of ciliary action.

In making these observations it was customary to employ a suspension of powdered carmine, following the displacement of the particles by the cilia with a binocular or compound microscope. When desirable, these observations were supplemented by direct examination with a water-immersion lens.

[1] A brief preliminary report on a portion of the material contained in this paper was published in the Proc. Amer. Soc. Zoöl., Anat. Rec., vol. 34, p. 116.

This investigation was suggested by Dr. R. G. Harrison and conducted under his guidance. The writer wishes to acknowledge his deep obligation to him.

NORMAL CILIARY ACTION OF AMBLYSTOMA PUNCTATUM

The youngest embryo on which ciliary action was noted was at stage 18,[2] just before the closure of the neural folds. The action was very weak and restricted to a small area on the outer cranial portion of the neural folds. More often, the action is first indicated just after the neural folds close (stage 19–20). Figures 1 to 3 suffice, without any verbal description, to illustrate the pattern of action at representative stages of development. The stronger action in later stages, indicated by the use of heavier arrows, is due to increase in number and length of the cilia. Viewed in profile, the ciliated cells in the older embryos were seen to be elevated above the neighboring non-ciliated ones.

On 3-cm. larvae examined, the ciliary action had become quite weak except on the gills, where a strong distally directed action persists until metamorphosis.

DETERMINATION OF POLARITY OF CILIATED CELLS

Woerdeman ('25), working with Rana esculenta embryos, argued that the direction of ciliary beat, very constant in this species, must be controlled either by, 1) factors in the ciliated cell itself; 2) factors in the cell's environment (through relation of cell to body axis, e.g.), or, 3) influences from both of these sources. Operating on embryos of different stages of development, he cut out small squares of ectoderm, rotated them 180°, and reimplanted them. Later, the direction of beat on the graft was tested. In embryos operated before late gastrulation the cilia on the graft beat normally, i.e., with those on the surrounding ectoderm; operated later, they beat in the opposite direction. From this it was concluded, 1) that the direction of beat is determined by the relation of the cell

[2] Stages refer to Harrison's unpublished normal series. Stages 15–19 cover the period during the closure of the neural folds.

Figs. 1 to 3 Embryos at stages 22, 30, and 40, respectively. The arrows indicate the direction of the water currents produced by the ciliary action.

to the embryo as a whole (i.e., by the cell's environment), but that, 2) once determined, the direction of beat is independent of factors environmental to the cell. Determination thus consists in the localization or restriction to the individual cell of the factors governing direction of beat.

In addition to the work of Woerdeman, there are the earlier and somewhat similar experiments of von Brucke, Merton, and Isayama. Von Brucke ('17) and Merton ('23 a) rotated pieces of ciliated epithelium from the roof of the mouth of the adult frog, and Isayama ('24), pieces of epithelium lining the trachea of the dog and the cat. Their results agree with those of Woerdeman; the cilia of the transplanted pieces do not adjust their action to the normal condition. These experiments, however, have no bearing on the determination problem.

The author has repeated Woerdeman's experiments on Amblystoma punctatum. Operating on embryos of various stages of development, pieces of ectoderm were extirpated, rotated 180°, reimplanted, and allowed to heal in. After ciliary action became strong, the direction of beat on the graft was tested with carmine particles. The results show that in this species determination generally occurs during the closure of the neural folds, considerably later than in the species examined by Woerdeman. The cilia developing upon ectoderm transplanted before this stage beat in the same direction as the cilia of the adjacent ectoderm (fig. 11); transplanted after this stage, in the opposite direction (fig. 9). The time of determination slightly precedes the first appearance of cilia.

Effect of development at low temperature on time of determination of ciliary polarity

The cases operated may be divided into two groups: 1) embryos developed before operating at room temperature or slightly below and, 2) embryos developed at retarded rate at a temperature of 7°C. An embryo of group 2, operated when the neural folds had just closed, let us say, is accord-

ingly considerably older in point of actual time than an embryo of group 1 operated at the same stage of development.

The results as tabulated (table 1) show that determination comes relatively earlier in embryos developed at low temperatures. While at stage 18 determination is infrequent in the embryos developed at room temperatures, it has occurred in the majority of the 'cold-room' embryos of the same stage. Further, in the latter group, some embryos were found determined as early as stage 16. No operations were performed

TABLE 1

Group I, embryos developed before operating at room temperatures. Group II, embryos developed before operating at 7°C. Data contained in table show that determination of ciliary polarity comes relatively earlier in embryos developed at low temperature

	GROUP I		GROUP II	
	Cases determined	Cases undetermined	Cases determined	Cases undetermined
Stage 16			3	8
Stage 17	0	4	1	2
Stage 18	1	7	10	3
Stage 19	8	1	23	0
Stage 20	5	1		
Stage 21	6	0		

on embryos younger than this, but it is quite possible that some of these would show determination. Ciliary action also becomes initiated earlier in the embryos developed in the cold.

We see that the effect of the low temperature has been to selectively inhibit morphological development, while permitting the determination of ciliary polarity to proceed at a somewhat more rapid rate. This carries a suggestion to the experimenter that 'ice-box' material may not always be entirely dependable for operative work.

Relation between determination of, 1) ciliary polarity and, 2) 'organ-forming fate' of the embryonic material

Spemann ('18) found that, if pieces of prospective epidermis and neural plate were interchanged by transplantation at the beginning of gastrulation, both pieces were still capable of developing into tissues appropriate to their new position. Pieces transplanted at later stages, however, differentiate according to their origin. The 'organ-forming fate' of these portions of the embryonic material is, therefore, determined during gastrulation. It may now be pointed out that Woerdeman demonstrated that the polarity of the ectodermal cells is also determined during gastrulation. Additional experiments by Woerdeman strengthened his belief ". . . . dass die organbildende Funktion der Ectodermzellen und ihre Polarität entweder gleichzeitig oder wenigstens mit sehr geringem Zeitintervall determiniert werden." In A. punctatum the author has shown that the polarity of the ectodermal cells is not determined until the neural folds are about to close. In this species, then, the organ-forming potencies are not only evidently localized, but are partly realized before the ectodermal cells acquire their polarity. Apparently, the two processes of determination bear no fixed relation to each other in different species.

Transplantation between embryos with and without ciliary polarity determined

We have seen that the ectodermal cells receive their polarity during the closure of the neural folds, about stage 18–19. To ascertain the fate of cells which are permitted to skip this critical period, pieces of ectoderm were grafted, rotating them 180°, from embryos at stage 13–15 to older embryos at stage 20–22.

In the sixteen cases examined the action on the graft was at first quite evidently irregular, without unified plan. In most of these this condition had not been overcome in the late tail-bud stages (30–34) of the host. However, one could

usually discern at least a slight tendency toward regulation to the normal condition as the embryos approached this age, in three cases eventually amounting to virtual coordination of the action with that of the host.

These results emphasize the importance of the polarizing influence which the embryo exercises upon the ectoderm during the 'critical period' in determination. The tendency toward regulation to the normal condition suggests further, however, that the influence is not completely lost immediately after this more crucial stage.

Pieces were also transplanted from older (stage 20–22) to younger (stage 13–15) embryos, to see if the polarity previously given the graft by the donor can be reversed when the graft, rotated 180°, is later subjected to the polarizing influence of the host.

As before, the results form a graded series. When first examined, practically all of the grafts showed some influence of the host, in the form of irregular ciliary action. In part of the cases this evidence was slight, the original polarity remaining predominant as the host grew older. In others, however, the polarity received from the donor became less marked, and in six cases was completely reversed by the time the host had reached stage 25–30. In these embryos the action on the graft was entirely coordinated with that of the host. A few cilia had already developed on most of the grafts before they were transplanted. Presumably, it is these same cilia, with others developing later, which come to beat in the opposite direction.

It should be explained here that even on rotated grafts transplanted between embryos both with ectodermal polarity determined, the action near the borders is not always in harmony with the polarity of the graft as a whole. This is possibly due to the shifting of cells during healing, rather than to any influence of the host. At any rate, the manifestations in the present experiments were much too striking to be invalidated by such considerations.

REVERSAL OF CILIARY ACTION

Reversal of ciliary action is not uncommon in the Infusoria, and flagellar reversal has been reported recently for Volvox by Mast ('26). Among metazoans, however, relatively few cases have been encountered. Probably the most complete account is that given by Parker ('96, '05 a and b), who described ciliary reversal in Metridium marginatum. He found that reversal of stroke could be induced by application to the mouth of the animal of such substances as potassium ion, crab-meat juice, creatine, and a few other organic materials found in meat. Since the ciliary action of a resting animal is directed outward over the lip of the mouth, normal feeding probably often necessitates reversal. Carlgren ('05) describes a similar condition for certain other actinians. Parker emphasizes that reversal is always a purely local phenomenon, occurring only in areas to which the stimuli are directly applied. Chemically inert substances, such as paraffin, he found ineffective in changing the direction of beat. Torrey ('04), however, reported that such mechanical agents cause reversal in Sagartia davisi.

Purkinje and Valentin ('35), Valentin ('42), and Englemann ('68) reported spontaneous reversal of the cilia on the gills of the mussel. Merton ('24), without citing the authority, states that ciliary reversal has been observed in appendicularians, aiding in the expulsion of undesirable food substances. The few other cases of reversal, all among small invertebrate forms, have been reviewed by Parker ('05 a). Certain flatworms, notably, possess special ciliated areas which may exhibit variability in direction of beat.

Nervous control of ciliary action has been reported recently for a few forms,[3] and Merton ('24) contends that reversal is always a manifestation of such regulation. He would thus class reversal as one of the spontaneous or voluntary responses of the organism. The bearing upon this generalization of the present study will be discussed later.

[3] Merton ('23 a), Carter ('26), and Grave and Schmitt ('25).

Roux ('95, p. 106) makes incidental reference to a young frog embryo which reversed the direction of its rotation within the egg capsule between succeeding observations. One cannot be entirely sure from his description that the change in direction of rotation was caused by reversal of the cilia. With this possible exception, I am unaware of any reference to reversal of ciliary action among vertebrate embryos or animals.

The writer has found that in embryos of Amblystoma punctatum reversal of ciliary action may be quite readily induced by application of the proper mechanical stimuli. Those found effective were: intimate contact of the epithelium with a foreign surface, e.g., the floor of a wax or glass dish; immersion of the embryo in a dense, resistant medium; contact with the egg membranes in which the embryo develops. Only a few observations on the effect of chemical stimuli have been made thus far, and will not be reported in this paper.

A. Reversal due to contact of ciliated epithelium with a foreign surface

It had been noted in transplantation experiments that when slightly arched glass rods, or 'bridges,' were placed over the head of the embryos to hold grafts in place until they heal, the embryos sometimes moved posteriorly from under the bridge. Normal ciliary action would carry the animal anteriorly against the bridge. To study this response, several embryos (stages 25–31) were subjected to conditions similar to those just described. The embryo was laid on its side in a small depression in the floor of a wax dish, and a bridge placed on its head (fig. 4). The size of the depression and the adjustment of the bridge insured intimate contact of one side of the embryo with the bottom of the dish. After a time the embryo moves posteriorly from under the bridge and swims backward over the floor of the dish. If proper adjustment is made, this response can be invariably induced. It is always accomplished by reversal of ciliary action on the side of the embryo next the dish (fig. 5). Even when embryos

are allowed to lie free in the dish, in about 2 to 4 per cent of the cases their own weight causes sufficiently intimate contact with the floor to affect the ciliary action in the area exposed, altering the direction of beat.

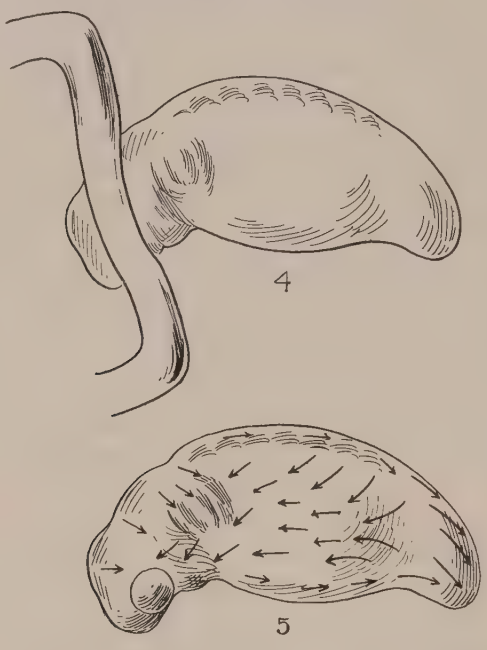


Fig. 4 Arched glass rod ('bridge') placed on head of embryo, which lies in small depression in the dish. Cilia on opposite side of embryo (next the dish) reverse under these conditions, carrying the embryo posteriorly from the bridge.

Fig. 5 Represents the condition developing upon the embryo shown in figure 4, on the side lying next to the dish. The cilia reverse their action, carrying the embryo posteriorly from under the bridge on its head.

Reversal occurs only on the part of the embryo exposed to contact with the dish or bridge. The purely local nature of this phenomenon, indicated throughout the different experiments, was demonstrated in the following way. A small round hole was made in the bottom of the usual depression in a wax dish, so that when the embryo was placed in the depression, the area of ectoderm immediately overlying the hole was free from contact with any surface. A bridge was then

placed on the head of the embryo. After a time, when the bridge was removed, the ciliary action of the ectoderm which had been suspended over the hole showed quite normal ciliary action, while on the portion of the trunk which had been in contact with the dish, the action was reversed or otherwise abnormal.

The approximate time required for reversal after a bridge is placed on the embryo, recorded for about sixty cases, averages twenty minutes. Of the sixty cases, slightly over one-half required fifteen minutes or less to reverse, averaging eleven minutes. About five minutes is the minimum time in these experiments. The response, then, is not immediate.

Normal ciliary action is eventually restored after the bridge is removed from the embryo. When the area showing reversed ciliary action was left next the dish, the average time for forty cases was about eighty minutes, with individual cases varying from twenty-five minutes to four hours, and probably longer in a few cases which could not be followed for a greater time. In fact, one often gets the impression that the preference of normal over reversed action is remarkably slight if the conditions are arranged at all suitably. When the area showing reversal was removed from contact with the dish, complete resumption of normal action usually required about one to two hours.

The behavior of the cilia during a change in the direction of beat is different under the two conditions specified, namely, when the epithelium is in contact with the floor of the dish and when it is freely exposed in the water. When next the dish, the direction of beat during reversal or resumption of normal action seems to swing through a half-circle, the ciliary action remaining coordinated. This concerted, gradual shifting through 180° of the direction of ciliary beat is more readily followed during the change from reversed back to normal action by observing the changing direction of movement of the embryo over the floor of the dish.

Contrastedly, when the area showing reversal is freed from contact with the dish, the ciliary action does not remain co-

ordinated during resumption of normal action. The first signs of irregular activity may appear after about fifteen or twenty minutes. Any coordination persisting later becomes limited to smaller areas, so that in one region the cilia may still beat anteriorly after in another they have already resumed normal action by swinging through an arc of 180°.

Contact with a surface is effective, then, not only in inducing altered action, but also in coordinating the beat of the cilia. However, the pattern of action is sometimes atypical. The case illustrated in figure 6 may be offered as an example. The specific conditions under which this vortical pattern of action becomes induced have not been investigated, but one

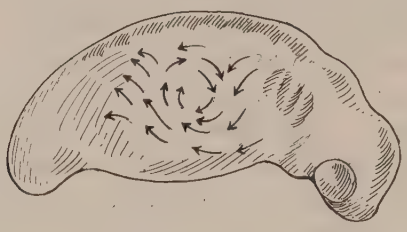


Fig. 6 'Vortical' pattern of ciliary action, occasionally developing on side of embryo next the dish after a bridge has been removed from over the middle of the trunk region. The embryo spins slowly around on a stationary transverse axis.

may sometimes observe such behavior, on the side of the embryo next the dish, after a bridge has been removed from the middle of the trunk region. The bridge taken away, the embryo spins around on its side on a stationary axis, as one might expect. Although of odd pattern, the action cannot be said to lack unified plan.

Direction of beat not controlled by position of bridge upon the embryo. The behavior of the embryos in escaping posteriorly from a bridge on the head might at first suggest that reversal is a specific response to the position of the bridge. In a few cases, however, it was found that reversal was induced, although with more difficulty, when a rod was placed on the posterior extremity of the embryo (in this experiment it is necessary to use a rod with roughened sur-

face to prevent it from sliding from the sloped contour of the tail). Moreover, although reversal is at first induced by a bridge on the head, it does not persist if the bridge is permitted to remain there. Examination at short intervals reveals that the direction of beat is constantly changing, often swinging back and forth between the normal and reversed condition or describing a complete arc of 360°.[4]

The effect of the position of the bridge was tested in a third way. After reversing the ciliary action by a rod on the head, the rod was immediately shifted to the tail. It was interesting to find that the reversed action rapidly became normal again, the time required in forty cases averaging only fifteen minutes. One may compare this with the eighty minutes necessary for resumption of normal action when the rod is not placed upon the tail.

This need not indicate, however, that a rod on the tail is specific for posteriorly directed action. Reversed ciliary action carries the embryo against a rod on the tail, just as the action when normal propels it against a bridge on the head. In both cases the cilia, in tending to carry the embryo under the rod, bring it more firmly against the dish, thereby increasing the intimacy of the contact of the epithelium with the dish. Consequently, it is not surprising that the time required for a change in the direction of beat is approximately the same in both cases (about fifteen minutes). Reversed action rapidly becomes normal when a rod is placed on the tail, therefore, because of the intimate contact with the dish thus achieved, and not because of any specific effect of the

[4] When a bridge is left on the embryo for a few hours, the ciliary action of the side next the dish may become quite weak, and sometimes uncoordinated. With the embryo in such intimate contact with the dish, it is possible that there is a limited oxygen supply available, especially since the greater effort of action may increase the normal oxygen requirement. According to Gray ('24, p. 112), "The oxygen appears to remove some factor which is the direct result of activity and which, if allowed to accumulate, will inhibit the activity of the whole ciliary apparatus." The author has found that ciliary action ceases when embryos of A. punctatum are left in oxygen-free water for some time. This is not preceded by any marked sign of abnormal action, however, so it is unlikely that reversal itself is directly related to oxygen metabolism.

position of the rod. One is also enabled to account for the difficulty, already indicated, of inducing normal action to reverse by placing a rod on the tail. Here the normal action strives to carry the embryo away from the rod (instead of under it and thus against the dish, as when the rod is on the head), and, accordingly, the pressure of the epithelium against the bottom is slight. All the evidence indicates, then, that the direction of beat is not determined by the position upon the embryo of the mechanical agent. We can say merely that reversal is a response to contact with a foreign surface.

Merton's ('24) contention that reversal is a manifestation of nervous regulation of ciliary action has already been mentioned. It is true that reversal in actinians and appendicularians at first suggests voluntary control of the cilia used in feeding. In the former group, however, Parker ('05 a), on the contrary, interprets the evidence as indicating freedom from such nervous intervention. In Amblystoma embryos there is no possibility of nervous regulation, for the cilia are reversible as early as the late neurula stage, considerably before any nerves develop. This fact and the experimental results make it clear that in no sense can reversal be regarded as a spontaneous or voluntary response in this form.

B. Effect of dense medium on ciliary action

We have just seen that intimate contact of the ciliated epithelium with a foreign surface produces a change in direction of beat. We may conceive for present purposes that this influence operates in one of two ways: first, through pressure upon the epithelial cells proper, or, secondly, through the resistance offered by the foreign surface to the free activity of the cilia themselves.

To test these possibilities, several embryos were placed in the jelly obtained by soaking quince seeds in water. A dense medium of this kind offers resistance to the ciliary action, presumably without exerting undue pressure upon the epithelium.

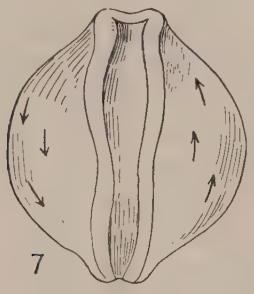
The embryos were usually left in the quince-seed jelly overnight, then removed, and examined with powdered carmine. Some rather striking cases of altered ciliary behavior were noted. In several embryos (stages 25 to 34) the ciliary action exactly girdled the embryo. On the right side, let us say, the cilia beat directly toward the midventral line; on the left side, directly toward the middorsal line. The current thus formed was uninterrupted under the ventral surface of the embryo below and over the neural ridge above. This general type of activity, encountered more frequently than any other, would tend to rotate the embryo on its longitudinal axis. Such a movement presumably encounters less resistance from the medium than any other, such as the normal forward progression, which would meet the full resistance of the dense medium.

These results are of particular interest. They indicate, first, that mechanical agents cause reversal through the resistance which they offer to the ciliary action, and, secondly, that the pattern of ciliary action may be in accord with factors involving the least resistance to the effective beat of the cilia.

C. Effect of vitelline membrane on ciliary action

Another series of observations falls directly into line with those just described. As is commonly known, the egg and early embryo is enclosed within a very thin vitelline membrane, closely apposed to the surface of the embryo. After about stage 19 or 20, however, the membrane ceases to be tight-fitting, either breaking down or increasing in diameter. Further, the gelatinous capsule swells as the embryo grows older, so that soon the embryo has the freedom of a rather spacious cavity. Consequently, the ciliary activity is but little hindered by any confining influence, and the embryo is propelled around this chamber. Most of the observations to be described, however, were made on embryos whose development had been retarded by low temperature. It has been pointed out that such embryos may exhibit ciliary ac-

tivity relatively earlier than embryos developed at normal temperatures. Accordingly, before the embryo is free of the close-fitting vitelline membrane, a rather strong ciliary action may be developed. The embryos are hence in much the same situation as embryos immersed in quince-seed jelly; their ciliary activity is resisted at almost all points by the closely apposed vitelline membrane. The resulting behavior is much the same under both conditions.



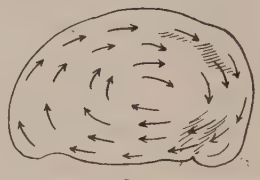


Fig. 7 Illustrating effect of close-fitting vitelline membrane (not shown) on the ciliary action of young embryos still resting in the upright position. This action, which girdles the embryo horizontally, rotates it on a stationary dorsoventral axis.

Fig. 8 Illustrates effect of vitelline membrane (not shown) on ciliary action of embryos which have come to lie on the side. The embryo spins around on a stationary transverse axis in the direction opposite that indicated by the arrows showing the pattern of ciliary action.

Between stages 15 and 19, when the neural folds come together, the back of the embryo rests uppermost. About stage 19 or 20 the embryo, becoming elongated and laterally compressed, comes to lie on its side. The type of rotation in the vitelline membrane depends on which of these two positions the embryo occupies. If the back is uppermost, the embryo rotates on a dorsoventral axis, with the cilia beating anteriorly on one side and posteriorly on the other (fig. 7); anteriorly on the right side, if rotation is to the right, on the left side if rotation is to the left. Observations on embryos between stages 20–22, which have come to lie on the side, revealed two types of rotation. Some rolled over and over

on a longitudinal axis and exhibited the girdle type of ciliary action already described for embryos in quince-seed jelly. Others lay on one side and spun slowly about on a fixed transverse axis; these showed the general pattern of ciliary action illustrated in figure 8. A single embryo, as it develops from stage 18 to 22, has been observed to exhibit, in the order given, each of the three types of rotation.

One sees how well the pattern of ciliary action is accommodated to the condition of the embryo. Embryos with open neural folds, e.g., if turned over, always show a strong tendency to resume the upright position, with the heavily yolked belly lying down. The girdle type of action, which tends to rotate an embryo on its longitudinal axis, would therefore encounter the opposition of gravity in these young embryos. The only type of ciliary action which will permit free rotation is that described, where the cilia beat anteriorly on one side and posteriorly on the other (fig. 7). When the embryos have reached stage 20, however, the girdle action, or the ciliary action shown in figure 8, encounter the least opposition from gravity. At this time the embryos are elongated and lie on their side, so that rotation according to the type of action in figure 7 would obviously be difficult.

These observations again strongly suggest that the pattern of ciliary action established is in accord with factors involving the least resistance. To induce altered behavior of any type, of course, this resistance must reach the minimal value required to overcome the natural tendency toward normal action. A thin mixture of quince-seed jelly, e.g., produces little if any change in the normal ciliary action.

D. Ciliary reversal in other species of amphibians

The observations on embryos of other species were not extensive, but reversal of ciliary action was noted for each form examined, viz., Amblystoma tigrinum, the axolotl (ordinarily regarded as a variety of Amblystoma tigrinum), Rana pipiens and Rana palustris, and Pleurodeles waltli. Of these, reversal is most difficult in Rana and the axolotl. In the

former, the only cases noted were caused by contact with the vitelline membrane, in embryos in the neurula stage. Reversal was induced with difficulty in a few axolotl embryos by placing them under glass weights. A medium of quince-seed jelly caused reversal in Pleurodeles[5] embryos; and contact with the dish was effective in Amblystoma tigrinum. Probably in none of these forms is reversal so readily induced as in Amblystoma punctatum.

These additional observations indicate that ciliary reversal is not unusual in amphibian embryos.

EFFECT OF CURARE ON CILIARY ACTION

Amblystoma punctatum embryos at the stage when muscular motility first appears (stage 34) were immersed in a curare solution, judged by its action when injected into adult frogs to be of effective strength. Presumably because of its poor penetrating power, the drug required two to three days to paralyze this motility. About a day later, the ciliary action usually lost its coordination, diverse but constant currents occupying areas of varying extent. The ectoderm generally remained healthy and the ciliary action unusually strong. Removed to fresh water, motility returned, but the cilia remained uncoordinated for the next few days, when the ciliary action became so weak that its pattern could not readily be ascertained.

No interpretation of these results is at present justified. Certainly, they offer no basis for assuming that in these later stages ciliary coordination is under nervous regulation. Curare-injected embryos of Pleurodeles showed no similar response. It is quite likely that the curare acted directly on the ciliated cells, interfering in some way with their normal physiology. The results seemed sufficiently striking, however, to deserve mention.

[5] These eggs were laid in the laboratory by animals imported from Germany. The axolotl eggs were also obtained from animals in this laboratory.

COORDINATION OF CILIARY ACTION

In these experiments square pieces of ectoderm were transplanted, rotating them 180°, between stained and unstained embryos in which the direction of ciliary beat had already been determined; accordingly, the cilia of the graft beat

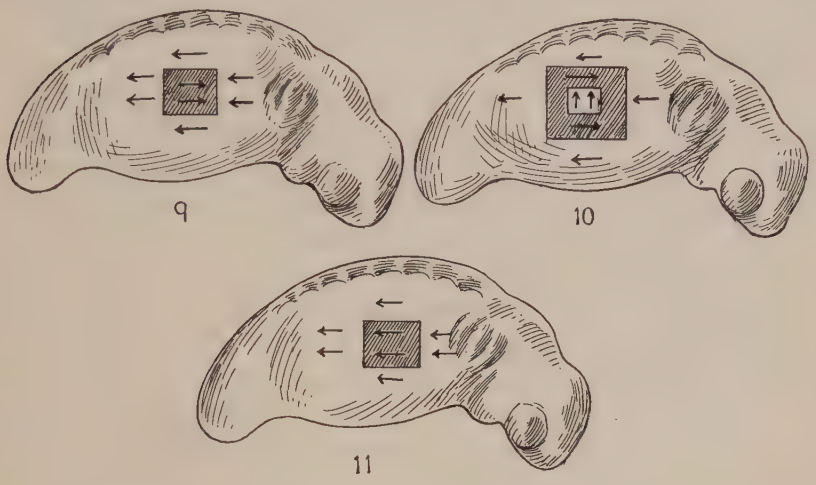


Fig. 9 Illustrates ciliary action of graft rotated (180°) after closure of neural folds (i.e., after determination of ciliary polarity). This condition is evidenced only when the operated side is free from contact with a foreign surface.

Fig. 10 Double operation. The large graft is rotated 180°; the smaller piece grafted into its center is rotated 90°. The action on both grafts changes to beat with the cilia on the surrounding host ectoderm, when this side of the embryo is placed next the dish.

Fig. 11 A graft has been rotated 180° and then the operated side of trunk placed next the dish. In response, the cilia on the graft reverse to beat with the cilia of the host, as the figure shows. The figure also represents the condition when rotation of the graft is performed before closure of the neural folds (i.e., before determination of ciliary polarity).

oppositely to those on the host (fig. 9). In a few cases the grafts were quite large, comprising about one-half the area of ectoderm on one side of the trunk. Into the center of two of these larger grafts were implanted smaller pieces of ectoderm, rotated only 90° (fig. 10).

The operated side of the embryo was then placed next the dish and retained there for some time. When examined later,

the ciliary action of the graft, or of both grafts in cases such as shown in figure 10, had become well coordinated with the action on the remainder of that side of the trunk (fig. 11). Ordinarily, the cilia beat posteriorly, i.e., normally, but in a few cases the action (on both graft and host ectoderm) was directed anteriorly or toward the middorsal or midventral line. Anterior action could be induced by placing a bridge on the head.[6]

If the operated side is now retained in a position so that it does not come into contact with the dish, the original condition is eventually restored (figs. 9 and 10). This procedure may be repeated a number of times with the same embryo. The results on coordination are clearest if the experiments are performed on younger embryos, about stage 28 or less. As the embryo grows older, reversal, and consequently coordination of ciliary action, becomes more difficult, possibly because of increasing differentiation of ciliary or cell structure. In similar experiments with axolotl embryos, in which reversal is induced with considerable difficulty, results negative to those just described for Amblystoma punctatum were obtained.

These experiments, with those already described, show strikingly how contact with a resistant surface or medium coordinates the ciliary action.

Seat of factors regulating ciliary action. Contact of the epithelium with a foreign surface produces changes resulting in coordination of ciliary action. To determine whether these changes are restricted to the epithelium or originate from the deeper layers of the embryo, the following experiment was performed. Over a large piece of ectoderm, spread out on a wax dish with ciliated surface uppermost, was placed a

[6] Practical application can be made of ciliary action in confirming the orientation of grafts. If, however, the species being used is one in which ciliary action is reversible, it is obvious that precaution must be taken to prevent contact of the operated area with the dish for some time before the test is made. If a graft escapes while being transferred to another animal, the writer has found it feasible to rediscover its orientation by placing a few mesoderm or yolk cells on it from the wound and watching their displacement.

small piece of very thin cover-slip. When this was removed a few minutes later, all the cilia of the sheet of ectoderm were beating in a direction at right angles to the normal. In this case any possible influence of the deeper layers upon the beat was entirely eliminated. We see, accordingly, that the ciliary phenomena described in this paper are purely epithelial in origin and nature, hence independent of any control of the embryo as a unit.

Nature of the coordinating mechanism. It has been shown that the changes brought about by contact originate in and are confined to the epithelium. In attempting to interpret their nature in terms of some physiological mechanism cap-

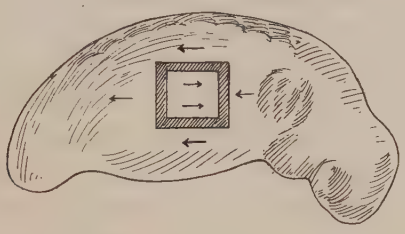


Fig. 12 Graft rotated 180°, but not permitted to heal at its borders with the host ectoderm. Shaded area around graft represents gap between it and the surrounding epithelium. The cilia on the graft nevertheless reverse to beat with those on the host when this side is placed next the dish.

able of coordinating the ciliary action, there occurs to one the 'neuroid' type of stimulus (Parker, '19)—a transmitted impulse which travels in the epithelium. A stimulus of this kind would require an uninterrupted path. In testing this possibility, accordingly, the following procedure was employed. Square pieces of ectoderm were cut out, rotated 180°, and reimplanted. They were not allowed to heal at their border, however; instead, the gap around the graft was increased by pushing back the cut border of the surrounding ectoderm (fig. 12). After merely pressing upon the graft for a short time to insure that it remain in place, the operated side of the embryo was placed next the dish. In about ten or fifteen minutes, the action on the graft had become well co-

ordinated with that of the remaining ectoderm on this side. A broad band of the underlying mesoderm was visible entirely around the graft, so that there was no possibility of contact between the border of the graft and the surrounding ectoderm. If coordination is effected by a transmitted stimulus, one would have expected failure of coordination when the path was broken, as in this experiment.

It might be objected that when the ectoderm is interrupted by a cut, the underlying mesoderm layer may be substituted for the transmission of a common impulse between the graft and the surrounding epithelium. Experiments where the cut extended through both ectoderm and mesoderm were few in number and were not checked sufficiently to exclude this possibility entirely. They strongly indicated, however, that such is not the case.

We have just seen that the ciliary action of individual cells becomes coordinated when they are not contiguous, and thus probably unable to share with each other a common stimulus. It is likely, therefore, that the changes responsible for concerted action arise independently in each cell. In illustrating how this might operate, let us consider the case where a piece of ectoderm has been rotated 180° (fig. 9), and the operated side of the embryo then placed next the dish. Under this condition, it is quite conceivable that the resistance offered the ciliary action of the graft by the opposed action of the surrounding ectoderm produces conditions within the cells or cilia of the graft which can be relieved only by reversal of beat. Thus the individual cells within the area showing coordination would arrive independently, without the aid of a special coordinating mechanism, at the same physiological state.

The cilia of an area in contact with a surface possibly beat together, accordingly, because of the sensitivity with which the direction of beat responds to the resistance developed when their action is other than concerted. Recalling the observations on embryos in quince-seed jelly or within the vitelline membranes, one may see that the cilia do have a tendency

to beat in the direction which encounters the least resistance. The unified action of the cilia in the experiments with the jelly showed further that a foreign surface, which might conceivably convey a polarizing or coordinating influence, is not requisite to concerted action. All the evidence suggests, therefore, that coordination of ciliary action is not effected by a transmitted impulse, whether carried by the epithelium or the surface with which it lies in contact. Further work aims to test the validity of this view.

The changes responsible for reversal may not involve the interior of the cells, possibly being restricted to the cilia or the cell surface. The nature of the changes, whether 'electrical,' e.g., is not indicated by these experiments.

Experimentally induced coordination of ciliary action should not be confused in type with the coordination exhibited under normal conditions. In the latter case the action is concerted merely because the cells in any given area have all acquired the same polarity at determination. In these experiments, however, there was superimposed a coordinating mechanism capable of temporarily changing the normal polarity of the ciliated cells.

This mechanism, imposed by a resistant surface or medium, is, as we have seen, epithelial, purely involuntary, and probably not a transmissible stimulus. It is suggested that the experimentally induced coordination and reversal phenomena are merely manifestations of the tendency of the cilia to beat in the direction which encounters the least resistance.

SUMMARY AND CONCLUSIONS

1. Cilia first appear on the ectoderm of embryos of Amblystoma punctatum about the time that the neural folds close. As described for other species of amphibians, the major currents produced by their action are directed caudally.

2. The polarity of the ciliated cells is determined during the closure of the neural folds. The cilia of grafts rotated (180°) before this stage beat in the same direction as the cilia of the adjacent ectoderm; transplanted later, in the opposite direc-

tion. Occurring at this relatively late stage, determination of ciliary polarity obviously does not coincide with the determination of 'organ-forming fate' of the embryonic material (i.e., the localization in the embryo of epidermis- and neural-plate-forming potencies). This is in contrast to the condition in Rana esculenta (Woerdeman, '25).

3. Low temperature evidently retards morphological development more than the process of determination, for ciliary polarity is established at an earlier stage in embryos developed in the cold. This shows that in development the determinative and morphological phases are, to a limited degree, independent of each other. It also warns against the dependability of 'ice-box' material for experimental work.

4. Pieces grafted from stage 13–15 embryos to older ones at stage 20–22, thus skipping the critical period of development of the embryo during which determination occurs, manifest in their action a lack of polarity. This may gradually be overcome by the host, however. On the other hand, determined grafts, transplanted in reverse orientation on younger embryos, may have their determination reversed by the influence of the host.

5. Temporary reversal of ciliary action may be induced by application of mechanical stimuli. Effective agents or stimuli are: intimate contact with a foreign surface, such as the floor of a dish; a dense, resistant medium, and contact with the vitelline membrane. These stimuli are effective probably through the resistance which they offer to the activity of the cilia. The evidence indicates that the cilia beat in the direction in which they encounter the least resistance. In no sense can reversal be regarded as a 'voluntary' response. It is a purely local phenomenon, occurring only in areas to which the stimuli are directly applied.

6. In reversing, the ciliary action seems to swing coordinately through an arc of 180°.

7. Reversal of ciliary action is also reported for embryos of Amblystoma tigrinum, Rana palustris and Rana pipiens, Pleurodeles waltlii, and the axolotl. Ciliary reversal has not been reported previously for vertebrate animals or embryos.

8. Embryos immersed in curare solution for an extended period of time finally lose muscular motility. Abnormal ciliary action usually follows. However, the results do not justify the assumption that the ciliary action is regulated in any way by nervous control.

9. Intimate contact of the epithelium with a foreign surface has a striking coordinating effect on the ciliary action. The cilia of a graft rotated 180° reverse to beat with those of the surrounding area, when the operated side of the embryo is placed against the floor of the dish. It is suggested that this is due to the resistance offered the action on the graft by the opposed action on the surrounding ectoderm.

10. The changes responsible for coordination and reversal are apparently restricted to the epithelium itself, since they occur when the epithelium is isolated from the underlying layers.

11. Coordination of ciliary action under the experimental conditions described is evidently not effected by a transmitted stimulus traveling in the ciliated epithelium. The cilia of a graft rotated 180° reverse to beat with those of the surrounding ectoderm, even when a cut separates the two areas.

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3998

Effects of Experimental Block of the Amphibian Nervous System.

J. S. NICHOLAS.

From the Osborn Zoological Laboratory, Yale University.

Experiments previously reported[1] have shown that the cut ends of the spinal cord can be prevented from reuniting by the removal of a comparatively large amount of the nervous system and the implantation into the wound of the anlage of the limb and pronephros.

The responses of such an embryo during early stages are strictly in keeping with the findings of Coghill and Harrick. The uninjured portions of the nervous system behave as individual units and take up their function with a very slight delay over the normal. As the animals differentiate, a complex series of reactions occurs by means of which stimuli are transmitted across the gap in the nervous mechanism. This transfer of stimuli takes place through the intermediation of the musculature which is in that region.

The first integration of response was noticed in the reactions occurring after stimulation in the head region. The sensory pick-up is mediated through the uninjured medulla giving rise to a motor response through the 7th cranial nerve which activates the gular musculature and the arcuate muscles of the gills. This reaction is transmitted to the anterior trunk region where the sensory pick-up acts in the pectoral region to transmit the stimulus to the uninjured cord of the trunk. This motivates the musculature of the flank and tail and gives rise to distinct swimming movements.

The limbs which have been transplanted into the wound area develop synchronously with the normal limbs. They are generally single and although they develop in close proximity to the gills, show no trace of the developmental dominance of that tissue. In

[1] Nicholas, J. S., *Anat. Rec.*, 1928, xxxviii.

every case the laterality of these limbs has been that of the side from which they were taken.

The innervation of the transplanted limbs is through the 7th nerve. They possess to some degree all the primary movements found in the limb in this form. For a long period after the operation, the transplanted limb moves only when there is movement of the gills, but in later stages the limb is capable of flexion and extension of the whole limb or any of its parts without perceptible motion in the gills.

Other authors have stated the conditions which may limit the movement of transplanted limbs,[2] and have shown that function may be limited by the morphological deficiencies of the transplanted parts as well as due to nervous deficiencies. The present experiments show that a transplanted limb even with a very abnormal nervous supply is capable of having all the primary movements The amount of movement, however, is quantitatively incomplete.

[2] Detwiler, S. R., *J. Comp. Neur.*, 1925, xxxviii.

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4011

Further Studies on the Life History of Blepharisma Undulans.

LORANDE LOSS WOODRUFF.

From the Osborn Zoological Laboratory, Yale University.

Studies on the life history of *Blepharisma undulans* were carried on for just 6 years, all the animals employed being the progeny of a single individual isolated November, 1921. The work was planned to determine, in particular, the viability of this infusorian in the absence of fertilization, and the effects of fertilization on the rate of reproduction, in an attempt to broaden the results from our earlier studies on *Paramecium aurelia*[1] and *Spathidium spathula*.[2] A preliminary statement of the characteristics of this race of *Blepharisma undulans* was published in 1922,[3] and a summary of the chief experimental results was presented in 1927.[4]

The present communication is to reiterate the results previously presented in the light of the final year of study of the culture.

In brief, the conclusions derived from *Paramecium* and *Spathidium* that these infusorians are capable of unlimited reproduction, under favorable environmental conditions, without recourse to fertilization, may now be extended to *Blepharisma;* and, furthermore, the conclusion that fertilization accelerates the division rate, as previously demonstrated in *Uroleptus*[5] and in *Spathidium,*[2] may now be extended to *Blepharisma.*

These studies have been aided by a grant from the Bache Fund of the National Academy of Sciences.

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[1] Woodruff, L. L., *Quar. Rev. of Biol.*, 1926, i, 436.

[2] Woodruff, L. L., and Spencer, PROC. SOC. EXP. BIOL. AND MED., 1921, xviii, 240, 303; *J. Exp. Zool.*, 1924, xxxix, 133.

[3] Woodruff, L. L., and Spencer, PROC. SOC. EXP. BIOL. AND MED., 1922, xix, 339.

[4] Woodruff, L. L., PROC. SOC. EXP. BIOL. AND MED., 1927, xxiv, 769.

[5] Calkins, G. N., *J. Exp. Zool.*, 1919, xxix, 121.

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4013

Normal Survival Period Following Bilateral Epinephrectomy in the Cat.

E. L. COREY. (Introduced by J. S. Nicholas.)

From the Osborn Zoological Laboratory, Yale University.

Marked discrepancies in regard to the normal survival period in cats, following bilateral adrenal extirpation, are apparent throughout the literature dealing with the adrenal cortex. Zwemer[1] states the average survival period for a series of 43 cats to be 53 hours with 26 and 111 hours as the extremes. Swingle[2] finds the average survival period for bilaterally epinephrectomized cats to be about 60 hours. Houssay and Lewis[3] found that animals from which the last vestige of cortical tissue had been removed survived but from 24 to 26 hours. Hartman,[4] in seeking an active cortical extract, considered 90 hours as a marked prolongation of the life of bilaterally operated cats. The above references will serve as evidence that no definite criterion has as yet been established in work of this nature.

In a series of 31 animals the author has found the average survival period to be 115 hours, and concludes that the normal life-span of untreated bilaterally epinephrectomized cats must be placed slightly in excess of 100 hours. These findings have been completely corroborated by the recent work of other investigators.

Hartman[5] has placed the average survival period at from 5 to 6

[1] Zwemer, R. L., *Am. J. Physiol.*, 1927, lxxix, 641.

[2] Swingle, W. W., *Am. Nat.*, 1927, lxi, 132.

[3] Houssay, B. A., and Lewis, J. T., *Am. J. Physiol.*, 1923, lxiv, 512.

[4] Hartman, F. A., PROC. SOC. EXP. BIOL. AND MED., 1926, xxiii, 467.

[5] Hartman, F. A., MacArthur, C. G., and Hartman, W. E., PROC. SOC. EXP. BIOL. AND MED., 1927, xxv, 69.

days. Marine and Bauman[6] also agree that the life-span should be placed at somewhat in excess of 100 hours (5.3 days). Professor Swingle, in recent communications to the author, states that he would place this period in excess of 100 hours. Zwemer, in a recent conference with the writer, has stated that he would at present place the life-span of control animals at approximately 100 hours.

The author is inclined to attribute the comparatively short survival periods obtained by various workers to imperfections in operative technique, excessive visceral manipulation during operation, or to excessive handling and excitation.

The establishment of the normal survival period of bilaterally epinephrectomized cats is of prime importance, since the efficacy of all treatment devised to prolong life, as well as the potency of extracts obtained from the adrenal cortex, can only be interpreted by comparison with a definitely established normal survival period.

[6] Marine, D., and Bauman, E. J., *Am. J. Physiol.*, 1927, lxxxi, 86.

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4014

Growth of Heteroplastically Transplanted Eyes and Limbs in Amblystoma.

VICTOR C. TWITTY AND JOSEPH L. SCHWIND.
(Introduced by J. S. Nicholas.)

From the Osborn Zoological Laboratory, Yale University.

For the purpose of comparing the growth of normal and heteroplastically transplanted organs, eye vesicles (with lens ectoderm) and limb discs were transplanted between *Amblystoma punctatum* and *A. tigrinum*. The operations were done in the "tail-bud" stage, and the grafts were placed in the normal position and orientation. To standardize the feeding, both species were daily fed the maximum amount they would eat.

Measurements of normal and transplanted eyes were made with a micrometer ocular at regular intervals. As accurate measurements of the developing limb are impossible, pairs of animals with heteroplastically transplanted limbs were killed from time to time as a comparison.

Measurements made as soon as the outline of the eye became sufficiently visible, before either species had reached the feeding stage, showed that the normal and transplanted eyes were of the same size. This relation is maintained during most of the larval period. As the time of metamorphosis approaches, accurate measurements of the eye can no longer be made, but apparently the normal *tigrinum* eye now becomes slightly larger than the transplanted eye on the *punctatum*, and the transplanted *punctatum* eye becomes larger than its control. In other words, the 2 eyes on the *tigrinum*, normal and transplanted, become larger than the 2 on the *punctatum*.

In normal development, the fore-limb of *Amblystoma punctatum* becomes visible externally about the same time as the gills, while

that of *A. tigrinum* does not appear until just before the animal begins to feed. When the limb disc is transplanted heteroplastically, the transplanted limb and the normal limb of the donor species appear at the same time. During the major portion of larval life the transplanted limb and the normal limb of the donor species maintain the same size. Eventually, in late larval life, the normal limb of the *tigrinum* becomes somewhat larger than the limb transplanted to the *punctatum,* and the transplanted *punctatum* limb becomes larger than its control. The growth relations of the eye and limb after heteroplastic transplantation are therefore the same.

There is a great difference in the growth rate of the 2 species. With maximum feeding, 5 weeks after operation the *tigrinum* has attained a length twice that of the *punctatum,* and therefore requires much more food. We have found that if this greater food requirement of the *tigrinum* is not met, its growth will be correspondingly retarded, and the normal *tigrinum* organ will be smaller than that transplanted to *punctatum,* and conversely the *punctatum* organ on *tigrinum* will not become as large as the normal control on *punctatum.*

With maximum feeding, then, the behavior of heteroplastically transplanted organs indicates that the factors controlling their growth are intrinsic to the grafted tissue.

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4022

Notes on Some Parasites from Chimpanzees.

HAROLD KIRBY, JR. (Introduced by L. L. Woodruff.)

From the Osborn Zoological Laboratory, Yale University.

Through the kindness of Dr. H. C. Bingham and Professor R. M. Yerkes an opportunity was presented to examine for parasites 4 chimpanzees maintained by the Institute of Psychology at Yale University. These animals came from various parts of Africa and have been at the laboratory of the Institute since September 12, 1925, before which time 2 of them were for 3 months in a zoo in Philadelphia.

Since March, 1926, occasional samples of stools from the chimpanzees have been examined, always with the result of finding in large numbers motile forms of a *Balantidium* morphologically indistinguishable from *Bal. coli*. Cysts occurred only very rarely. Although these ciliates have swarmed in the intestines of the animals, at least since examinations first began, and have been passed in great numbers, presumably in every stool, the chimpanzees are healthy and show no visible signs of suffering from their infection. The stools have been more often soft than well formed, and have occasionally been diarrhoeic, but consistency has varied with the animals' diet. In stools kept at room temperature the ciliates have remained alive for several hours, some as long as 32 hours after leaving the host.

Balantidia in anthropoids have been studied by Brooks,[1] Noc,[2] Brumpt,[3] Joyeaux,[4] Christeller,[5] Hegner and Holmes,[6] Ziemann[7]

[1] Brooks, H., *Proc. N. Y. Path. Soc.*, n. s., 1903, iii, 28.

[2] Noc, F., *C. R. Soc. Biol.*, 1908, xliv, 878.

[3] Brumpt, E., *C. R. Soc. Biol.*, 1909, lxvii,, 103.

[4] Joyeaux, C., *Bull. Soc. Path. Exot.*, 1913, vi, 612.

[5] Christeller, E., *Virchow's Arch. Path. Anat.*, 1922, ccxxxviii, 396.

[6] Hegner, R. W., and Holmes, F. O., *Am. J. Hyg.*, 1923, iii, 252.

[7] Ziemann, H., *Arch. Schiff. Tropenhyg.*, 1925, xxix, Beih. 1, 434.

and Cunha and Muniz.[8] As a result of their observations it appears that the ciliates may parasitize orangutans, macaques, capuchins, baboons and chimpanzees; and that the animals are in most cases carriers, not apparently suffering from the presence of the parasites, but may in some cases develop ulcerative dysentery. Dobell and O'Connor state that these animals react to *Balantidium* in much the same way as does man and Hegner[9] says "it has long been known that the organism is pathogenic to primates." Most of the authors have supposed that the species with which they dealt was *Bal. coli.* Hegner and Holmes, however, point out some differences between the *Balantidium* of *Cebus variegatus* and *Bal. coli,* while Cunha and Muniz describe a new species, *Balantidium aragaoi,* from *Cebus caraya* in Brazil. Brumpt parasitized a macaque *(Macacus cynomolgus)* with *Balantidium* from a pig, and Walker[10] infected monkeys with ciliates from both pigs and man. Ziemann twice tried to infect himself by ingesting trophozoites and cysts from chimpanzees, but was unsuccessful.

In these animals, as well as in man, *Balantidium* may sometimes invade the tissues and produce intestinal ulcers. The ulcers have been studied, and in them ciliates demonstrated, by Brumpt, Walker, Noc and Christeller. In many of Walker's experimentally parasitized monkeys the tissues were not invaded. Several fatal cases of balantidiasis in anthropoids have been reported. Thus a family of orang-utans in the New York Zoological Park (Brooks), a macaque at the Pasteur Institute (Noc), and two chimpanzees at the Berlin Zoological Gardens (Christeller, Ziemann) were said to have died of the disease. Diarrhoeic symptoms have frequently been found associated with the presence of *Balantidium,* without evidence that the ciliate was the cause of the condition. Ziemann suggests that infested animals may for a long time be carriers, until *Balantidium* becomes actively pathogenic through lowering of the host's resistance or change in the pH of the intestinal mucus. In the chimpanzees at Yale there are heavy infections apparently unaccompanied by pathological conditions.

Besides *Balantidium* 2 other Protozoa have been found in these chimpanzees. The ciliate, *Troglodytella abrassarti,* was very abundant in fresh feces when the animals were first examined, but a few weeks later it became scarce and during many subsequent examinations was not seen. Recently, 20 months after the first observation, there were still a few present in 2 of the animals. *Troglodytella* perishes much more quickly than does *Balantidium,* espec-

[8] Cunha, A. M. Da, and Muniz, J., *C. R. Soc. Biol.,* 1927, xcvii, 823.

[9] Hegner, R. W., *Arch. Path. Lab. Med.,* 1927, iii, 1009.

[10] Walker, E. L., *Phil. J. Sci.,* 1913, viii, sec. B, 333.

ially at room temperature, but a few have remained alive after eight hours in feces kept at 36° C.

In the mouths of 2 chimpanzees a species of *Entamoeba* was found. This closely resembled *E. gingivalis,* and was especially remarkable for its eruptive manner of forming pseudopodia, a characteristic which has seldom, if ever, been recorded for *E. gingivalis.* In the infected mouths there were evidences of pyorrhea, with pockets in which the amoebae were plentiful. Several attempts to cultivate them in the media used for *E. gingivalis* were unsuccessful.

In addition to the Protozoa mentioned above, several intestinal worms have been present. A fragment of a cestode, *Bertiella studeri* (determined by C. W. Stiles) was passed by one ape when the examinations first began, and in the faeces of another eggs of *Trichuris* and *Ascaris* were found.

Herr Harrison:

On the status and significance of tissue culture[1]).

The fact that animals are made up of parts, each with a certain rôle to play in the economy of the whole, has been recognized from the very beginning of biological science and was long ago made familiar in the fable of the belly and the members. Modern anatomy and physiology are still based largely upon this fundamental concept, for although in recent years we have heard much of the organism as a whole, living creatures are not studied so much in their entirety as by processes of analysis in which each organ or part is taken, as far as possible, by itself.

Very early in the history of biology it was pointed out that the parts of animals are of two kinds. Thus Aristotle in his Historia Animalium[2]) says: „Of the parts of animals some are simple: to wit, all such as divide into parts uniform with themselves, as flesh into flesh; others are composite, such as divide into parts not uniform with themselves, as, for instance, the hand does not divide into hands nor the face into faces . . . . All those parts that do not subdivide into parts uniform with themselves are composed of parts that do so subdivide, for instance, hand is composed of flesh sinews and bones".

This distinction, which characterizes the difference between what we now call tissues and organs and which is expressed in language scarcely less precise than that of the biology of to-day, is said to have antedated even Aristotle and to have been borrowed by him from Anaxagoras.

The same idea is formulated again by Aristotle in „The parts of Animals"[3]), in which he recognizes three degrees of composition:

1. Composition out of the elements [protoplasm and cells].

2. Composition of the parts uniform with themselves, such as bones flesh, ligaments [tissues out of cells].

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1) Address given in abriged form at the opening session of the Tenth International Zoölogical Congress in the Hall of the Hungarian National Museum, Budapest, Sunday, September 4, 1927.

2) Translated by D'Arcy W. Thompson. Oxford. The Clarendon Press.

3) Book 2, Chap. 1.

3. Composition of the parts not uniform with themselves [organs out of tissues].

Handed down through the ages, this idea became of greater significance through the revelations of the microscope in the seventeenth and eighteenth centuries, but it was not until Bichat published his Anatomie Générale at the outset of the nineteenth century that accurate systematic decriptions of the tissues were given. Bichat, however, worked without the aid of the microscope and enumerated as tissues twenty one different structures, including many now recognized as more complex. It remained, therefore, for Schwann[1]) in the cell theory to lay the foundation of modern histology. Since then this branch of biologic science has divided its attention fairly between the investigation of the simple tissues and that of the more complex organs, and its very name places emphasis on the former rather than on the latter.

Physiology, on the other hand, busied itself more with the larger entities of the body. Its methods were better adapted to the study of organs than to that of the more recondite functions of cells and tissues. Nevertheless Claude Bernard[2]) based physiology on the concepts of cellular differentiation, division of labor, and autonomy of the cellular elements, all subordinated to the whole by means of the milieu intérieur, and modern general physiology, following the principles so lucidly stated by that great master, has sought to study directly the activities of protoplasm and cells. Still the work in this field has depended upon the use of cells that are naturally isolated, such as unicellular organisms, blood cells and the eggs of higher forms. The technique for a general study of cells and tissues was lacking.

The discovery that it is possible to isolate and keep growing in culture media very small fragments of almost any tissue either of embryonic or of adult organisms has in some measure supplied this need and has opened a new field now generally known as „tissue culture“ or „explantation“, through which, it is hoped, a deeper insight may be gained into what is meant by the autonomy of the structural elements and their relations to their milieu.

The expression „tissue culture“, which has come into such general use and which has been employed in the title of the address assigned to me to-day, is in some degree a misnomer, for it fails to convey in full the idea it represents. Moreover the use of the phrase might be taken to imply that this method of research — in reality a technique — is an object in

1) Mikroskopische Untersuchungen, 1839.

2) Leçons sur les phénomènes de la vie. Paris 1878.

itself, whereas it is but a means of investigating the properties of cells under conditions not previously available. What was originally the designation of a method has in fact come to connote the field of knowledge it has brought to light.

The mode of experimentation in question has been employed in the study of both plant and animal tissues and has proved to be of particular value in the fields of embryology, histology, physiology, pathology and immunology, but in spite of the wide divergence of the lines in which it has been applied, there is a realm of common interest, apart from mere technique, that holds together those who work with it and has brought here to-day this large group of investigators. This common ground centers about an interest in the activities of protoplasm, cells and tissues, as such, and rests upon the recognition that what goes on in isolated cultures gives some measure of information regarding living processes within the organism itself, and further, that the reactions of living elements in disease are not fundamentally different from the normal processes of development and function. Our method affords a means of studying these reactions where conditions are most simple.

The methods of tissue culture have been mothered by various necessities. In the case of my own work on the development of the nervous system[1]) there was need for an experiment in which the behavior of certain cells could be observed when removed from the bewildering conditions obtaining within the embryonic body, for it was recognized that the ordinary methods of histology were inadequate to answer definitely the question of the origin of the nerve fiber. Here, then, was a problem that demanded for its solution some such radical form of isolation as that afforded by the method of explantation.

Others approached the field in conscious search of a physiology of cells and tissues, and in this, as usual, the botanists anticipated the zoölogists. Thus, Haberlandt, who was the first to cultivate isolated cells of higher organisms, sets forth very fully in his introductory paper the purposes and possibilities of modern tissue culture[2]). On the animal side

1) Proc. Soc. Exp. Biol. and Med., Vol. 3, 1907 (Also in Anat. Rec., vol. 1); Anat. Rec., vol. 3. 1908; Journ. Exp. Zoöl., Vol. 9, 1910.

2) „Es sind meines Wissens bisher noch keine planmäßig angelegten Versuche gemacht worden, isolierte vegetative Zellen von höher entwickelten Pflanzen in geeigneten Nährlösungen zu kultivieren. Und doch müßten die Ergebnisse solcher Kulturversuche manches interessante Streiflicht auf die Eigenschaften und Fähigkeiten werfen, die die Zelle als Elementarorganismus in sich birgt, sie müßten Aufschlüsse bringen über die Wechselbeziehungen und gegenseitigen Beeinflussungen, denen die Zellen innerhalb des vielzelligen Gesamtorganismus ausgesetzt sind.“ Sitz.-Ber. Mat.-Nat. Kl. Kais. Akad. Wiss. Wien, Bd. 111, Abt. 1, 1902, p. 69.

Leo Loeb, in reviewing his earlier work said: „It [tissue culture] will I hope, ultimately lead to the building up of a physiology and pathology of tissues in a similar sense as we now possess a physiology and pathology of organs“[1]). A similar thread has run through the work of Carrel[2]), who more consistently and perseveringly than any one else has aimed to go beyond the mere explantation of fragments and to carry on for long periods true cultures of cells in pure line.

The introduction of a method as radical as this was naturally met with skepticism on the one hand and with, perhaps, an oversanguine enthusiasm on the other. These opposite attitudes may still be occasionally observed, even though the skeptics have been met at most points and the enthusiasts have often encountered disappointment. The method has thus fairly established itself and the number of investigations involving its use has increased at a greatly accelerated rate within the past few years. Articles touching upon some phase of the subject are appearing at frequent intervals and are attracting wider and wider interest. In the latest bibliography published[3]), covering the years 1925 and 1926, about four hundred titles are listed; a number of extended treatises on the subject in at least four languages have appeared; and the journal devoted to this field and founded by Professor Rhoda Erdmann less than three years ago, has already achieved signal success. All of this, taken together with the wide application of the method in biology and medicine, makes it clear that the subject has extended far beyond the bounds that any one individual may reasonably be expected to cover in a short address. I must, therefore, plead my natural limitations, if, in what follows, I should seem to ignore fields of work with which I have had no experience, and confine myself to the consideration of a few topics in the fields of embryology, histology and physiology where tissue cul-

1) Anat. Rec., Vol. 6, 1912, p. 120. About the time that Haberlandt's first paper appeared Loeb published his experiments with pieces of epithelium from the Guinea pig, imbedded in small blocks of clotted blood or agar which were placed for incubation in the body of another animal (Arch. Entw.-Mech., Bd. 13, 1902; Journ. Med. Res., Vol. 8, 1902). The technique was entirely different from Haberlandt's and had nothing in common with that of modern tissue culture, although the underlying purpose of the experiment was essentially the same. Loeb in an earlier paper, published separately (Über die Entstehung von Bindegewebe, Leukozyten und roten Blutkörperchen aus Epithel und über eine Methode isolierte Gewebsteile zu züchten, Chicago 1897), mentions having made experiments in which the agar blocks containing pieces of living tissue were incubated outside the organism. The results were not stated.

2) Journ. Exp. Med., Vol. 15, 1912; ibid., Vol. 17, 1913; ibid., Vol. 18, 1913; Physiol. Rev. Vol. 4, 1924.

3) Erdmann and Pinzke, Arch. f. Exp. Zellforsch., Bd. 4, 1927.

ture and related methods have proved fruitful and are promising of further progress.

The nerve elements.

The first observation[1]) on nerves growing in clotted lymph (the medium then used) gave ocular proof of the histogenetic theory of His[2]) and Ramon y Cajal[3]). The fiber was seen to be formed by the spinning of a thread of protoplasm by means of the amoeboid activity of the free end of a cell process. In this protoplasmic filament neurofibrillae develop a little later[4]). These findings have been confirmed and extended by numerous investigators[5]), and the degeneration of injured nerves and their subsequent regeneration in the same manner have also been followed in vitro[6]).

Subsequent studies by Levi[7]) have greatly enriched our knowledge of the physical properties and the physiology of the protoplasm constituting the growing fiber and have demonstrated the value of microdissection methods in work of this kind. According to Levi the young fiber is tough and resistant, and may be considerably stretched without suffering permanent injury. At the same time it is exceedingly sensitive to mechanical stimuli. If the cell body of a neuroblast is compressed, instantly its visible character changes and the nerve fiber attached to it is seen to emit fine processes, thus demonstrating its conductivity. Again the shock of cutting a fiber is transmitted to the growing end, as shown by the cessation of movement for a short time after such treatment. Subsequently the amoeboid activity is resumed and may continue for hours, during which time the fiber increases in length, as if it were still attached to the cell body. Ultimately, of course, this movement ceases and the severed fiber degenerates. Furthermore, both stumps of a cut fiber may manifest amoeboid activity and the two may reunite per primam intentionem, whereby the whole is restored and the distal portion spared the fate of degeneration. None

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1) Harrison, op. cit.

2) Abh. d. math.-phys. Kl. d. Kgl. Sächs. Ges. d. Wiss., Bd. 13, 1886; Arch. f. Anat. u. Physiol., Anat. Abt. 1887; Ibid., 1890.

3) Anat. Anz., Bd. 5, 1890; La Cellule, T. 9, 1892.

4) Burrows, Journ. Exp. Zoöl., Vol. 10, 1911.

5) Cf. Burrows, op. cit.; M. R. a. W. H. Lewis, Anatom. Rec., Vol. 6, 1912; Braus, Münch. med. Woch., 1911; Id. Verh. d. Ges. Deutsch. Naturf. u. Ärzte, 1911; Levi, Mem. R. Accad. Lincei, Vol. 12, 1917.

6) Cf. Braus, op. cit.; Ingebrigtsen, Journ. Exp. Med. Vol. 17, 1913 and Vol. 18, 1913.

7) Arch. f. exp. Zellforsch., Bd. 2, 1925.

of these facts could have been discovered by means of the more conservative methods.

From the time when the first nerve fibers were observed in culture it was evident that through the study of the effect of various physical and chemical agents on the outgrowth process we might gain insight into the play of factors that produce the complex pattern of the nervous system within the organism. It was soon shown that the outgrowth of a nerve fiber, like other cell movements, requires a solid substratum[1]), and this, taken together with observations on the course of nerves in the embryo, renders it likely that the configuration of the solid structures in the way of a developing nerve determines to a considerable extent its path[2]). In respect to responses to chemical stimuli, as postulated in Cajal's theory of chemotaxis, our knowledge is not so definite. Experiments made some years ago[3]), by a modification of Pfeffer's method, in implanting very small capillary tubes filled with various substances in proximity to the explanted nervous tissue gave conflicting results. Hoadley[4]) on the other hand, working with tissues implanted on the chorio-allantoic membrane, has found evidence that nerve fibers from pieces of the brain do tend to grow toward other pieces of tissue. Likewise the preliminary experiments of Ingvar[5]) point to the conclusion that nerve fibers respond as regards their direction of outgrowth to a very weak galvanic current. This, if definitely established, would afford important experimental evidence in favor of the theories Kappers[6]) and Bok[7]).

Connective tissue.

Cells from the connective tissue play an important, frequently a dominating part in tissue cultures of almost all organs. They survive without difficulty, remaining usually without marked visible differentiation,

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1) Harrison, Science, Vol. 34, 1911 and Journ. Exp. Zoöl. Vol. 17, 1914.

2) Journ. Exp. Zoöl., Vol. 9, 1910, p. 835.

3) Anat. Rec. Vol. 6, 1912, p. 192. Experiments made by the same method by Centanni and Ugurgeri (Accad. Fisiocrit. Siena, 1913) and later by Sanguineti (Rev. Patol. Nerv. e Ment., Vol. 19, 1914) are not relevant to this immediate question, since the outgrowths from pieces of peripheral nerves found by these authors are obviously, as they themselves admit, of connective tissue origin. Still this work, as well as the later study by Bisceglie (Arch. f. exp. Zellforsch., Bd. 2, 1926), is of great interest in showing that cellular outgrowth from explanted tissues is stimulated chemically, as indicated by the direction in which it takes place most actively.

4) Journ. Exp. Zoöl., Vol. 42, 1925.

5) Proc. Soc. Exp. Biol. a. Med., Vol. 17, 1920.

6) Psychiatr. en Neurolog. Bladen, 1916.

7) Psychiatr. en Neurolog. Bladen, 1915.

and lend themselves readily to indefinite culture. These cells are usually referred to as fibroblasts, but it has not been definitely proved that they have anything more than a passive rôle in the formation of connective tissue, and none of the numerous investigators who have cultivated them have proved them actually to form collagenous fibers. Baitsell[1]) has, in fact, brought forward strong evidence to show that, under certain conditions, connective tissue fibres may be formed by the transformation of the fibrin clot used in tissue cultures, just as scar tissue is formed in wound healing. The fibers would thus be a coagulation product rather than a direct secretion of the connective tissue cells. For this view much evidence has been advanced by those who have studied the histogenesis of connective tissue in the embryo[2]), for here fine fibrils formed out of a more homogeneous interstitial jelly seem to precede the immediate presence of cells. It is true that Levi[3]) described delicate fibers which were formed in certain cultures of mesenchyme from the heart and skin of advanced chick embryos, but their identity with connective tissue fibers is not claimed to be proved. Likewise M. R. Lewis[4]) has reported the formation of very delicate fibrillae in coverglass cultures of mesenchyme cells in purely fluid media, though in my opinion neither the exact nature of the fibrillae observed nor the direct participation of the cells in their formation has been shown. Thus there is not necessarily any inconsistency in the findings just referred to. It is likely that the further pursuit of the subject with the aid of improved technique will settle definitely this perplexing question. Certainly we may hope to find among the numerous investigators working with fibroblasts some one who will give us further information regarding the histogenetic powers of these cells.

Blood cells.

I hesitate to tread upon the difficult and hazardous ground of the formation and genetic relationship of the blood elements, for with all the diligence and ingenuity that has been spent in this field, uncertainty still exists with regard to even the most fundamental questions, and the enormous mass of publication that has been poured forth cannot possibly be

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1) Journ. Exp. Med., Vol. 21, 1915; ibid., Vol. 23, 1916.

2) Cf. v. Ebner, Zeitschr. f. wiss. Zool., Bd. 62, 1896; Merkel, Anat. Hefte, Bd. 38, 1909; Studnička, Anat. Anz., Bd. 31, 1907 and Bd. 40, 1911; Nageotte, C. R. Soc. Biol. Paris, T. 79, 1916; Id. L'Organisation de la matière etc., Paris 1922; Isaacs, Anat. Rec., Vol. 17, 1919; Baitsell, Am. Journ. Anat., Vol. 28, 1921; Id. Quart. Journ. Micr. Sci., Vol. 69, 1925; Harrison, Journ. Exp. Zoöl., Vol. 41, 1925.

3) Mon. Zool. Ital., Vol, 27, 1916.

4) Contr. to Embryol. Carnegie Inst. of Wash., Vol. 6, 1917.

mastered by an outsider. The work done by the method of tissue culture would alone fill several volumes, but, although it has made a definite impression and points in one direction, it cannot be said yet to have given a complete solution of the problems. However, the conditions obtaining within the organism are so complex that it is almost impossible to determine there the life history of cells capable of moving about by themselves and of assuming so many different guises. We must, therefore, have recourse to some such method of isolation as is afforded by tissue culture, where transformations may be followed by direct observation. For this reason it seems clear that this method will have the last word in clarifying the questions at issue.

The problems that confront us here concern the interrelations of the cells of the blood, bone marrow, lymph and connective tissue, together with their behavior both in the normal body and in reaction to conditions produced by disease. Various types of cells are known, differing from one another in size, shape, nuclear structure, granulation, motility and reaction to abnormal conditions, and occurring in the blood and tissues in relative numbers which fluctuate according to physiological and pathological conditions. Are these all merely different phases of one kind of cell or are at least two fundamentally different types involved which do not pass over into one another?

Work done with the aid of tissue culture methods generally favors the first of these alternatives. The old distinction between lymphogenous and myelogenous elements has been broken down by the discovery that cultures of the cortex of a lymph gland will yield a whole series of cells formerly thought to be characteristic of bone marrow[1]). The more recently drawn antithesis between monocytes and the various forms of leucocytes on the one hand, which are considered to be reticular in origin, and the clasmatocytes and erythroblasts on the other, thought to be derived from endothelium[2]), will also have difficulty in maintaining itself if the transformations which have been described by various investigators prove to be real.

All transitional stages between small lymphocytes, mononuclears and granular leucocytes and myelocytes have been observed[3]), and although the direct transformation of lymphocytes has not been actually followed in the living cell, the early findings of Maximow have been abundantly

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1) Maximow, Arch. Mikr. Anat., Bd. 97, 1923.

2) Sabin, Cunningham and Doan, Contr. to Embryol., Carnegie Inst., Vol. 16, 1925.

3) Maximow, op. cit.; Cf. also Arch. Russ. Anat., Histol., Embryol., T. 1, 1916; Awrorow u. Timofeewsky, Virchows Arch., Bd. 216, 1914.

confirmed by later workers[1]). The transformation of mononuclear blood cells into macrophages epithelioid cells and giant cells has been followed[2]), and it has been found that blood monocytes and tissue macrophages or clasmatocytes assume identical characters with regard to form, size, pseudopodial activity, mitochondria and granulations, when cultivated in the same media, and that these vary with the medium employed[3]). Again the change of mononuclears from cultures of blood over into fibroblasts has been repeatedly observed by many workers since first described by Carrel and Ebeling[4]). The occurrence of this transformation, which is of prime importance in the organization of blood clots in wound healing and which could hardly have been proved crucially by the older methods, shows that fibroblasts are one of the end products in the metamorphosis of blood cells. Recently, however, it has been found that under certain conditions, not yet altogether understood, fibroblasts may be changed back into macrophages[5]).

From all of this the inference may be drawn that the various forms of blood and connective tissue cells may be produced by the transformation of a monocyte-like element. Some of these transformations are known to be favored by certain qualities of the medium, as, for instance, the change of lymphocytes into myelocytes and granular leucocytes by the presence of bone marrow extracts[6]), and the hastening of the change of blood monocytes into fibroblasts through the action of sarcoma extract added to the culture medium[7]). From such facts we are naturally led to view the mode of activity of these cells and their particular habitus at any given time as responses to factors in the environment rather than as characters indelibly stamped upon them. It remains to be determined, however, which of the transformations are reversible.

Muscle and heart.

That the cells of the myotomes are self differentiating to the extent that normally striated fibers will develop from them in plasma was shown in my first work on the outgrowth of the nerve fiber in vitro. This was

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1) Cf. int. al. Fazzari, Arch. f. Exp. Zellforsch., Bd. 2, 1926; Caffier, Arch. f. Exp. Zellforsch., Bd. 4, 1927; Hirschfeld, ibid.

2) M. R. and W. H. Lewis, Journ. Am. Med. Ass., Vol. 84, 1925; Contr. Embryol. Carnegie Inst., Vol. 18, 1926.

3) Carrel and Ebeling, Journ. Exp. Med., Vol. 44, 1926.

4) Journ. Exp. Med., Vol. 36, 1922.

5) Fischer, Arch. f. Exp. Zellforsch., Bd. 3, 1927.

6) Maximow, op. cit.

7) Carrel, Journ. Exp. Med., Vol. 43, 1926.

in fact one of the essential control experiments, for in order to be sure that the interpretation of the nerve preparations was correct, it had to be shown that other tissues would undergo characteristic differentiations under similar conditions[1]). For the study of the histogenesis of muscle the methods of tissue culture are, however, of no particular value. On the other hand they have been an important adjunct in the study of the development of muscle function, especially that of the heart.

The pioneer work of Burrows[2]) showed that groups of partially differentiated myoblasts, which had migrated out of explanted pieces of embryonic chicken heart, might after some days begin to contract rythmically under cultural conditions that precluded contamination with any kind of nervous element[3]). Thus a further crucial test of the myogenic theory of the heart beat was given. An isolated cell was observed to beat with a rythm of its own and two portions of the cellular net, formed by the migrated cells, which had become functionally separated from each other by the degeneration of the intervening portion, pulsated still in the same rythm but with an alternation of phase, i. e. not synchronously.

Experiments made by M. R. and W. H. Lewis[4]) with different forms of contractile tissue of the chick embryo—smooth muscle from the amnion, heart muscle and voluntary muscle from the myotomes and limb buds—have shown that all may produce cells that extend into the medium and there contract rythmically. However, the cells which grow out from the explanted fragments undergo a considerable amount of morphological dedifferentiation, and while striated fibrillae are sometimes found, as, for instance, in some remarkable cases described by Levi[5]), this is the exception rather than the rule. Outgrowths formed from voluntary muscle often resemble the muscle buds formed in the regeneration of muscle in vivo, and similar buds growing from explanted mammalian heart muscle have also been described[6]). In culture, however, these buds usually remain protoplasmatic without characteristic fibrillae.

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1) Journ. Exp. Zoöl., Vol. 9, 1910, p. 812.

2) Science, Vol. 36, 1912; Münch. med. Woch., Vol. 59, 1912.

3) That fragments of heart from chicken embryos could pulsate in culture media was shown by Burrows in his first work (Journ. Exp. Zoöl., Vol. 10, 1911). Braus (Münch. med. Woch., 1911; Wiener Med. Woch., 1911) demonstrated the same for the heart of the amphibian embryo (Bombinator, Rana) and further that such fragments responded to electrical stimuli. Carrel (Journ. Exp. Med., Vol. 15, 1912) found pieces of embryonic chicken heart beating after sixty-five days of isolation in culture media.

4) Am. Journ. Physiol., Vol. 38, 1915; ibid., Vol. 44, 1917; Am. Journ. Anat., Vol. 22, 1917.

5) C. R. Assoc. Anat., 19. Réun., Strasbourg 1924.

6) Cf. Erdmann, Verh. Anat. Ges., Halle 1924.

Considerable discussion has taken place with regard to the nature of the cells which grow out from explanted heart tissue, some referring to them as fibroblasts while others, particulary Levi[1]), emphasize their myoblastic origin. It seems clear, however, from the work of M. R. and W. H. Lewis[2]) that both types of cells proliferate and migrate in cultures, myoblastic outgrowths occurring relatively more frequently from tissue taken from young embryos, than from older. Again, according to Erdmann, in preparations of the guinea-pig's heart myoblasts tend to predominate at first, but if the piece is replanted a few times with removal of the cells that have migrated, pure cultures of fibroblasts are obtained. Myoblasts seem therefore to be more active and fibroblasts to have greater powers of survival.

Pieces of embryonic heart planted out in culture media beat frequently with different rythm even when taken from the same individual and subjected as far as possible to identical conditions. Experimenting with such fragments, Fischer[3]) found that after two had established close organic union by outgrowth of cells from one to the other, which was completed after from 48 to 72 hours, the two pieces beat in complete unison. No such connection occurred between pieces from chick embryos and those from the duck. According to Olivo[4]), however, pieces of heart tissue from chicken and pigeon embryos do coalesce and in such cases complete synchronicity is established in the pulsation of the two components.

The mode of connection between the elements in cultures of cardiac muscle has been considered fully by Levi[5]) as part of the general question of the occurrence of syncytia. Many cells that appear fused with one another often move apart later, and contact between cells seems to be all that is necessary for the transmission of stimuli. Even in cells that are actually fused each nucleus seems to have a definite sphere of influence. Lewis[6]) takes a still more radical stand against the existence of syncytia, especially in the mesenchyne, with regard to which there had been perhaps most general agreement as to the reality of cellular fusions. The study of tissue cultures has therefore tended to restrict very much our notion of the real occurrence of syncytia.

In contrast to the foregoing, which concerns small fragments of heart tissue and isolated cells, the heart rudiment of the amphibian embryo,

1) Arch. Sci. Med., Vol. 50, 1916; Rend. Reale Accad. Lincei, Vol. 32, 1923.

2) Behavior of cells in tissue culture. In 'General Cytology' edited by E. V. Cowdry, Chicago, 1924.

3) Journ. Exp. Med., Vol. 40, 1924.

4) Arch. f. Exp. Zellforsch., Bd. 2, 1925.

5) Arch. f. Exp. Zellforsch., Bd. 1, 1925.

6) Anat. Rec., Vol. 23, 1922.

when removed from the body with a small amount of overlying ectoderm, will, as shown by Ekman[1]), develop into a heart having the main features of the normal organ. Sinus, atrium, ventricle and bulbus may be formed, the whole structure with a certain degree of curvature[2]) and enclosed in a cavity inside an epithelial sphere. Such hearts, which lack altogether a nervous system, contract rythmically and afford a valuable means for the study of the action of drugs[3]). Explanted without the covering ectoderm, the heart rudiment forms a pulsating organ, but it is less perfect in form than when it develops in a pericardial cavity inside an epithelial sack. Such aggregates, which are intermediate between tissue cultures proper and whole explanted organ complexes, are of great interest in relation to problems of organization and differentiation.

Explantation of organ rudiments into media and to the chorio-allantoic membrane.

The study of the development of organs has not been so greatly advanced by the method of explantation as that of histogenesis. Their growth is not perfectly correlated in the unusual environment of the plasma culture and, while normal differentiations do occur, the ensemble is usually atypical. The most readily interpretable cases are those in which the epithelial component of the organ rounds itself off into a covering for the rest of the cells, as in Ekman's hearts above referred to. Fischer's[4]) intestinal organisms, formed from small pieces of the gut of chick embryos are of this character, and show that at least the epithelium and connective tissue components undergo marked progressive differentiation. Explanted limb buds of chick embryos give rise to typical cartilage, connective tissue and epithelium according to Strangeways and Fell[5]) and under certain conditions they show in gross form the early stages of a normal limb skeleton. Typical differentiation of the retina occurs in explants of the

1) Oversikt. Finn. Vetensk.-Soc. Förhend., Bd. 63, 1920—21. Ann. Soc. Zool.-Bot. Fenn., Vol. 2, Helsingfors, 1924.

2) Stöhr, who has repeated and extended Ekman's experiments, emphasizes the defective nature of most of the explanted hearts and the dependence of this organ on its surroundings for the development of its normal form (Arch. Mikr. Anat. u. Entw.-Mech., Bd. 102, 1924; Verh. d. Phys.-Med. Ges. Würzburg, N.F. Bd. 50, 1925).

3) Cf. experiments by Schübel and Stöhr on heterotopically translpanted hearts. Arch. Exp. Pathol. u. Pharmakol., Bd. 104, 1924.

4) Journ. Exp. Med., Vol. 36, 1922.

5) Proc. Roy. Soc. London, B. Vol. 99, 1926.

optic vesicle of the chick[1]) and an optic cup with lens is formed when the amphibian optic vesicle is planted out with its covering ectoderm[2]). One of the most notable examples of the constructive development of organ units is that found by Rienhoff[3]) in the formation of tubules in explanted pieces of the mesonephros and permanent kidney of the chick embryo.

There is a procedure, akin to that of tissue culture that will probably prove to be of greater value in this field, even though it is of but limited application. This is the method of explantation upon the allantois of the embryo, whereby the explanted tissue is given a substratum from which it may obtain a continuous supply of nutriment through the circulation, and which, though living, has but very slight morphogenetic powers of its own and is very tolerant of intrusion.

First developed by Murphy and Rous[4]) for the study of transplantable tumors, this method has yielded interesting results with pieces of normal organs of either embryonic or adult animals. It has formed the basis of investigations on haematopoiësis[5]), on sex differentiation[6]) and on the phenomena of determination and differentiation of various embryonic organs[7]).

From all of these experiments it is seen that in the chick embryo at the stages studied most parts are capable of a high degree of self differentiation. According to Hoadley, for example, the primordium of the eye is already partly determined in the blastoderm of four hours incubation. More complete determination follows rapidly, as shown by the degree of development attained by grafts taken at closely succeeding stages. Again, the specific sex characters of the gonads are maintained, when growing on the allantois, in spite of the antagonistic sex hormones of the host that are present in the circulation when the latter is of opposite sex to the graft (Corinaldesi).

Removal from normal surroundings can, however, have a very different effect, and may bring to light unexpected powers of growth which

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1) Strangeways and Fell, Proc. Roy. Soc. London, B, Vol. 100, 1926.

2) Filatow, Arch. f. Entw. Mech., Bd. 107, 1926.

3) Bull. Johns Hopkins Hosp., Vol. 33, 1922.

4) Journ. Exp. Med., Vol. 15, 1912; Murphy, Journ. Am. Med. Ass., Vol. 59, 1912; id., (Journ. Exp. Med., Vol. 17, 1913.

5) Danchakoff, Anat. Rec., Vol. 10, 1916; id., Am. Journ. Anat. Vol. 24, 1918.

6) Cf. Minoura, Journ., Exp. Zoöl., Vol. 33, 1924; Willier, Journ. Exp. Zoöl., Vol. 46, 1927; Corinaldesi, Arch. f. Exp. Zellforsch., Bd. 4, 1927.

7) Danchakoff, Zeitschr. f. Anat. u. Entw. Bd. 74, 1924; Contr. to Embryol. Carnegie Inst., Vol. 18, 1927. Hoadley, Biol. Bull., Vol. 46, 1924; Journ. Exp. Zoöl., Vol. 42, 1925; ibid., Vol. 43, 1926. Murray and Huxley, Journ. Anat., Vol. 59, 1925; Brit. Journ. Exp. Biol., Vol. 3, 1925.

are ordinarily held in abeyance by the inhibiting action of the organic environment, as in the case of the pronephros. Normally undergoing regression and disappearing early in development, this organ when planted out on the allantois persists and grows materially [1]).

Microdissection.

The technique of micromanipulation [2]), thanks to the skill of Schouten, Barber, Kite, Chambers and Péterfi, has reached a state of almost incredible precision, which is commensurate with the delicacy of the objects it has to deal with. The adaptation of these methods to the study of cells in tissue cultures [3]) opens to view many interesting possibilities. It is feasible thus to investigate not only the physical properties of living cells and their constituent parts but also their reactions to mechanical, electrical and chemical stimuli locally applied, the effect of injecting foreign substances, and the consequences of definite mutilations.

On the other hand, it is doubtful whether the cell nucleus [4]) will tolerate much interference, as for instance the mutilation necessary to remove individual chromosomes. Its organization is so delicate that any attempt to manipulate by direct mechanical action the carriers of the genes is likely to fail. Such effects are for the present best obtained indirectly by the action of chemical and physical agents, such as ultra-violet light and x-rays, on the mechanism of cell division, whereby an abnormal distribution of the chromosomes to the two daughter cells may be brought about, as in the case of non-disjunction, or selective action upon individual chromosomes may be produced.

The prolonged culture of pure strains of cells.

One of the earliest and most persistent criticisms of the method of explantation was that tissues did not actually grow under such conditions but merely survived—that, in other words, there was no true tissue culture in the sense that we speak of the culture of bacteria [5]) Never well founded, this objection has been shown to be meaningless in so far

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1) Hoadley, op. cit.

2) For the history of the development of "micrurgical" methods see article by Péterfi in Abderhalden's Handb. Biol. Arbeitsmeth.

3) Péterfi, Arch. f. Exp. Zellforsch., Bd. 4, 1927.

4) Péterfi und Olivo, Arch. f. Exp. Zellforsch., Bd. 4, 1927.

5) Jolly, C. R. Soc. Biol. Paris, T. 69, 1910. Gargano, Publ. Staz. Zool. Napoli, Vol. 4, 1923.

as a large number of investigations are concerned, and furthermore, it has been completely refuted in other cases where it would have been of crucial importance. In the first place, many of the problems studied by the method do not require for their solution that the explanted tissue remain alive and reproduce indefinitely. This is quite obvious from the work on the outgrowth of the nerve fiber, on the histogenesis of other tissues, on the nature of the connections between cells, on the course of mitosis and in many other problems of cell structure and behavior. In the second place it is now known that permanent cultures are possible to a degree once thought to be altogether fantastic.

This was first demonstrated by Carrel and Ebeling[1]) with the now celebrated culture of fibroblasts from the heart of the embryonic chick, which was set out originally in February 1912 and is still growing with undiminished vigor. Since the beginning of the experiment the mass of growing cells has been divided and reimplanted usually at intervals of forty eight hours, in which period of time it about doubles its volume. The life of this line of cells has therefore exceeded by a number of years the span of life of an ordinary chicken and the amount of living material produced, potentially at least, has been far greater in mass than the body of a single individual. In fact had it been possible to provide nutriment for all the cells, and to allow them all to multiply, they would now greatly outweigh the terrestrial globe. The general significance of this indefinitely growing line of somatic cells, not to speak of the perseverence and skill required to obtain the result, renders this discovery the greatest single achievement in the field of tissue culture.

A number of other lines of cells, mostly avian, have also been grown for considerable periods of time in pure culture. This list includes mononuclear leucocytes[2]), cartilage[3]), and a number of different strains of epithelium, such as that from the iris[4]) and the thyroid gland[5]). Each type of cell, though losing some of its visible differentiation, nevertheless retains certain characteristics of form, mode of growth and physiological activity. Ebeling's culture of thyroid epithelium, after months of growth, formed follicles filled with colloid. Mammalian cells are also capable of

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1) Carrel, Journ. Exp. Med., Vol. 15, 1912; ibid., Vol. 20, 1914; Carrel and Ebeling, Journ. Exp. Med., Vol. 34, 1921; C. R. Soc. Biol. T. 90, 1924; Ebeling, Journ. Exp. Med., Vol. 17, 1913; ibid., Vol. 30, 1919; ibid. Vol. 35, 1922.

2) Carrel and Ebeling, Journ. Exp. Med., Vol. 36, 1922.

3) Fischer, Journ. Exp. Med., Vol. 36, 1922.

4) Fischer, Journ. Exp. Med., Vol. 35, 1922.

5) Ebeling, Journ. Exp. Med., Vol. 41, 1925.

indefinite cultivation, as shown by Erdmann's lines of connective tissue and endothelium from the guinea-pig embryo[1]).

As is now well known, the longevity of cell cultures can be extended beyond a short time only when the medium is properly constituted. A small piece of tissue left to itself in a drop of plasma, serum or artificial medium will cease growing actively after a few days, although some cells may remain alive for some weeks. If, however, embryonic tissue extract is added to plasma[2]) very active growth is set up which may continue indefinitely, provided the growing tissue is planted out again at short intervals or the medium is otherwise renewed.

In addition to their general interest, these strains of cells maintained over a long period of time are of great value in affording a uniform material, already acclimated to conditions of culture outside the body, for the analysis of the factors which influence the rate of growth. By measuring the halo of cells formed through the migration of elements from the implanted piece out into the surrounding medium the amount of growth may be determined to within an error of ten per cent or even less[3]), which is sufficiently accurate for use in a great variety of comparisons in which differences are large. Much has already been accomplished in this line, for the details of which those interested are referred to the original papers.

Differentiation, dedifferentiation and the influence of cells upon one another.

It will already have been gathered from what has been said that explanted tissues undergo a considerable amount of visible dedifferentiation. In fact some authors, as for instance Champy[4]), have gone so far as to claim that cells in culture revert to a primitive condition unless there are two types of cells side by side, for example connective tissue and epithelium, to react upon one another[5]). While a considerable body of evidence has been brought forward in support of this thesis, it can scarcely be maintained in last instance, for although cells under cultural conditions do lose some of their physical and functional characteristics, they nevertheless retain others, as shown by their form, habit of growth, behavior

1) Verh. Anat. Ges., Halle. Ergänz.-Heft, Anat. Anz. Bd. 58, 1924.

2) Carrel, Journ. Exp. Med., Vol. 17, 1913; ibid., Vol. 18, 1913. An interesting suggestion in this direction is made in Haberlandt's first paper (op. cit., p. 89—90).

3) Cf. Ebeling, Journ. Exp. Med., Vol. 34, 1921, and Carrel, Physiol. Rev., Vol. 4, 1924.

4) C. R. Soc. Biol. Paris, T. 75, 1913; ibid., T. 76, 1914. Arch. Zool. Exp., T. 53, 1913; ibid., T. 54, 1914; ibid. t. 55, 1915; ibid. T. 60, 1920.

5 Cf. Drew, Brit. Journ. Exp. Pathol., Vol. 4, 1923 (not seen in the original).

in various culture media and by their mode of reaction when recombined. Embryonic cells not only do not dedifferentiate immediately on explantation but exhibit marked powers of differentiation, as shown by the formation of nerve fibers, the differentiation of cilia and the cuticular border in explants of ectoderm and the formation of striated fibrillae in cells from the myotomes[1]). Notable examples of differentiation in tissues of more advanced stages are the formation of follicles filled with colloid in Ebeling's[2]) culture of thyroid and the complete course of spermatogenesis in cultures of the testis of the cecropia moth, as observed by Goldschmidt[3]).

While it may be accepted as demonstrated that pure lines of cells under culture do not by any means lose all of their differential characters, it is nevertheless true that mixed cultures behave differently from pure. It is this fact that has lent support to the more extreme views of Champy and Drew just referred to. Now this very circumstance is of essential importance in our study of organization. So long as we merely separate the different kinds of cellular elements found in the metazoan body and study their properties as isolated strains, we gain no deeper insight into the nature of those interactions that constitute organization. It is as if the chemist should content himself with obtaining pure substances and observing their properties, all the while neglecting the study of their reactions with one another. It is necessary here as there to investigate the phenomena of recombination — in other words to synthesize as well as to analyze[4]).

Certain things have already come to light that seem to me to be of significance in this direction. It is, for example, a very interesting and at present inexplicable fact that single somatic cells isolated in culture media do not proliferate. Experiments to this end made in my own laboratory some years ago but not published did not succeed and other workers[5]) have reported similar experience. As Fischer puts it, a colony of fibroblasts cannot arise from a single cell even when the nutrient conditions are most favorable. Likewise small groups of cells if isolated do not undergo division and their growth remains at a standstill. On the other hand certain tumor cells (Rous chicken sarcoma) are capable of multiplying and producing colonies when isolated singly.

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1) Harrison, op. cit.

2) Op. cit.

3) Proc. Nat. Acad. sci., Vol. 1, 1915; Arch. f. Zellforsch., Bd. 14, 1917.

4) Cf. Harrison, Anat. Rec., Vol. 6, 1912, p. 185.

5) Cf. Fischer, Journ. Exp. Med., Vol. 38, 1923; Arch. Mikr. Anat. u. Entw.-Mech. Bd. 104, 1925. Policard, C. R. Soc. Biol. Paris, T. 92, 1925; Laser, Arch. Exp. Zellforsch. Bd. 1, 1925.

The earliest and at the same time most extensive experiments on this question were made by Haberlandt[1]) with plant cells. It was found that while leaf cells of Lamium purpureum and hair cells of Tradescantia would undergo some growth, that is increase in size, when isolated singly, cell division never occurred. Experiments with small pieces of tissue from the potato showed a direct relation between the size of the piece or number of cells present within it and the number of cell divisions, and also that the presence of vascular bundles or sieve tubes stimulates cell division in the parenchyme of the explant. From his more recent studies Haberlandt[2]) has been led to attribute the power of inciting cell division to substances emanating from injured cells. These he terms wound hormones.

Again in the matter of differentiation such fragmentary experiments as we have indicate that it is stimulated or at least accelerated by the presence of two kinds of cells in close contact. The best example of this is found in the mixed cultures of pure strains of fibroblasts and epithelial cells in the experiments of Ebeling and Fischer[3]). Here a ten year old strain of fibroblasts was combined with a two months strain of epithelium. After cultivation together for some time it was found that the epithelium had rounded itself off into a sort of glandular tissue lying in a stroma of fibroblasts.

It will be possible undoubtedly to make further progress in the study of the interactions between different kinds of cells and thus bring to light various manifestations of dependent determination. That functional stimuli are transmitted from cell to cell in culture has been amply demonstrated, and it is hardly too much to expect of the method of explantation that it will give similar proof of the passage of formative stimuli.

Reconstitution of organisms from dissociated cells.

Leaving now the results of the more usual methods of tissue culture, we may next consider a somewhat different mode of isolation in relation to ontogeny and regeneration or reconstitution, for here, it seems to me, is a field that has been relatively neglected and one in which, through recombination of known elements, syntheses of viable organisms may be obtained more readily than by the combination of pure lines of cells from the most specialized animals.

1) Sitz.-Ber. Math. Naturw. Kl. Kais. Acad. Wiss., Wien. Bd. 111, Abt. 1, 1902; Sitz.-Ber. Kgl. Preuß. Akad. Wiss., Jahrg. 1913, 1. Halbbd.

2) Sitz.-Ber. Preuß. Akad. Wiss., Jahrg. 1921, 1. Halbbd.; Biol. Zentralbl., Bd. 42, 1922.

3) Journ. Exp. Med., Vol. 36.

It is well known that most organisms show some power of restitution of lost parts and that in some the whole can be restored by very small fractions, as in hydroids, some worms, acidians etc. This takes place either by the formation of a blastema at the wound surface, from which the missing parts develop or by a process of rearrangement of the constituents of the fragment (morphallaxis), as shown by Morgan[1]) years ago.

Now it has been found possible to go much further than the students of regeneration had done in the attainment of isolation by a method introduced in 1907 by H. V. Wilson[2]). If small fragments of certain marine silicious sponges are squeezed in fine bolting cloth, and the material which passes through is stirred into sea water, a fine suspension of isolated cells may be obtained, which after a time settle to the bottom. From this deposit small groups of living cells segregate and in due course reconstitute themselves into orderly arranged masses out of which new sponges develop. Some of these have been raised to sexual maturity.

The most important elements in this process are the archaeocytes (amoebocytes) which move about by amoeboid activity, unite by means of their ectoplasmic layer with other similar cells, incorporating also into the small masses thus formed other cellular elements of the sponge. There has been some difference of opinion between Wilson and those who have followed him with regard to the potencies of the different kinds of cells and the extent to which dedifferentiation occurs. According to Galtsoff[3]) the dissociated cells do not revert to an indifferent condition, as Wilson was inclined to think, but each retains some of its morphological characters, and no cell is totipotent. At least one kind of cell other than archaeocytes, namely pinacocytes, which constitute the dermal layer and the lining of the canals, is necessary to form a viable aggregate that will develop into a sponge. The only cells that probably do not take part in the reconstitution of the sponge masses are the collar cells, but in the calcareous sponge (Sycon) studied by Huxley[4]) the collar cells do enter into the composition of the masses and form the new flagellated chambers. Development of the restitution masses, according to Huxley, is primarily a process of sorting out, by which the dermal cells cover the surface and the collar cells pass to the interior. There are obviously important specific differences in the mode of reorganization but it is of equal interest,

1) Arch. Entw.-Mech., Bd. 9, 1900, p. 563—586; Regeneration, New York, 1901.
2) Journ. Exp. Zoöl., Vol. 5, 1907.
3) Journ. Exp. Zoöl., Vol. 42, 1925.
4) Phil. Trans. Roy. Soc. London, Vol. 202 B, 1912; Quart. Journ. Micr. Sci., Vol. 65, 1921.

whether brought about by redifferentiation of indifferent material or by local sorting of the elements.

It is interesting to note, as Wilson points out, that the archaeocytes have great regenerative power under other circumstances. They play the chief rôle in reproduction by means of gemmules and out ot them develop also the sexual cells. We have, then, in these cells elements which are most active in all three of the modes of reproduction occurring in sponges.

Another important fact brought out by these studies is that no coalescence takes place between cells of different species. Wilson has made mixtures of cells from two sponges that could be recognized by means of differences in pigmentation, but always with the result that each cell aggregate formed was of homogeneous origin. Galtsoff obtained similar results with mixtures of other species. This is odd in view of the fact that in vertebrates there is no difficulty in getting tissues of different species to unite and to grow and function in harmony, as in numerous well known cases of heteroplastic grafting in amphibian embryos.

Wilson[1]) later extended his experiments to the hydroids, showing that here, too, a similar power of reconstitution is present. In both Eudendrium and Pennaria now colonies of polyps grew out of masses formed by the reintegration of cellular sediment. The masses of cells readily come together, secrete a perisarc and later send out coenosarcal outgrowths on which typical polyps develop. Microscopic examination shows that at first a syncytial mass is formed in which cells of both ectodermal and entodermal origin are represented but without definite arrangement. Later an outer layer segregates, forming a definitive ectoderm and a more or less solid mass representing the yolk entoderm, which finally divides into a definite layer of entoderm closely applied to the ectoderm, and an irregular inner yolk mass, which gradually is used up. This stage closely resembles the planula stage in the normal ontogeny of Pennaria.

Experiments by Hargitt[2]) yielded similar results in Podocoryne and to some extent also in two species of Eudendrium, but in the latter the polyps, which are formed only in exceptional cases, are small and weak, and reconstitution usually does not go beyond the stages of encystment and stolonization[3], as appears always to be the case in

1) Journ. Exp. Zoöl., Vol. 11, 1911.

2) Biol, Bull., Vol. 28, 1915.

3) De Morgan and Drew (Journ. Marine Biol. Assoc., Vol. 10, 1914) repeated Wilson's experiments, using two species of Antennularia. Restitution masses were formed, some of which live das long as sixty days, but none of these went so far as to form polyps. Hargitt (op. cit.) reports similar failure in several species of hydroid polyps and medusae.

certain other hydroids. In Clava according to Föyn)[1], polyps are readily produced, the original sex of the colony being preserved, and hermaphroditic aggregates can sometimes be made by mixing cells from colonies of each sex,

Wilson again raises the question of the dedifferentiation of specialized elements, pointing out that the cell masses consist of both ectoderm and endoderm cells. The formation of the layered mass must take place, then, either by differentiation in situ irrespective of origin or by the wandering of of the original ectodermal elements to the surface. There is no histological evidence for the latter and Wilson therefore adopts tentatively the former view which means that already specialized cells may dedifferentiate and later become transformed into something else.

As far as is known this mode of reconstitution takes place only in sponges and hydroids. However, no exhaustive efforts have been made to find out to what extent in the animal kingdom it may occur. Wilson [2]) has made some experiments with the pressed out ovarian cells of the starfish (Asterias) and with cells from the stolon of Perophera, an ascidian, but with negative results. His experiments with Leptogorgia, a gorgonid, nevertheless lend encouragement to the view that under proper conditions successful syntheses may be obtained with the cells of this or related forms. It would seem worth while to experiment also with the regenerating tissues of forms in which the whole may be restored by very small fragments, such as the starfish Linckia, planarians, ascidians, Ctenodrilus, and some of the fresh water oligochaetes. By the use of this method, if further perfected, it will be possible to study not only the types of cellular combination that will produce new individuals but also to investigate the external physical and chemical conditions that influence the process. In the latter direction Galtsoff[3]) has already broken the way.

By employing dissociation in conjunction with the more usual procedures of tissue culture we may be able to study the transition between the process of reorganization and the simple propagation of cells in pure or mixed cultures without the production of individual organisms. Of much interest in this connection are the recent experiments of Murray[4]) in which it was found that small fragments of planarian tissue (P. dorotocephala) isolated in culture media would generally behave like tissue cultures and send out into the medium cells of various kinds. Some of the fragments, however, showed a certain amount of individuation either of an axiate or of a spherical type. Here, then, we have the actual

1) Zool. Anz., Bd. 61, 1924.
2) Journ. Exp. Zoöl., Vol. 11, 1911; Journ. Elisha Mitchell Soc., Vol. 39, 1923.
3) Journ. Exp. Zoöl., Vol. 42, 1925; Journ. Gen. Physiol., Vol. 10, 1926.
4) Journ. Exp. Zoöl., Vol. 47, 1927.

border-line between the apparent anarchy of an ordinary tissue culture and the subordination of elements requisite to produce a higher organization. The investigation of the conditions under which these different types of reaction occur should, as Miss Murray points out in her program, throw light on the whole question of reconstitution.

In fact much more should be done in cultivating invertebrate tissues. The work with vertebrates has, no doubt, been pushed not only because of the convenience of obtaining suitable culture media but also because of its greater practical and theoretical interest in medicine. Nevertheless, with the less specialized invertebrate elements we are more likely to obtain those syntheses which willh elp us to gain insight nto the meaning of what we call organization.

Relation to the general problems of embryology.

The relation of tissue culture to general questions of embryology has not been fully considered. With regard to the problem of determination, the method has served par excellence in proving that certain elements, such as muscle cells, neuroblasts and pigment cells are self determining so far as their histogenetic processes are concerned. On the other hand, as already pointed out, no adequate experiments have been made to show the interaction of cells in development, though enough has been found to show that here is a rich field for future study.

The determination problem has dominated experimental embryology ever since the first embryological papers of Roux and Driesch were published. To ascertain whether a given differentiation is due to activities inherent within the differentiating element itself, or whether it is dependent on influence from without, and if the latter, according to what pattern the actions take place, is a task that all will admit must, in some measure at least, take precedence over the study of the more hidden qualities of the activities themselves. However, the nature of the protoplasmic changes involved in determination and differentiation, and the differences between various cells in respect to the kinds of change they undergo are questions that demand equal attention if we are to give a rational description of development in general terms. The growth energy of different types of cells, changes in chemical composition as shown by vital staining and other microchemical methods, the nature of the changes going on in any transformation as suggested by the study of temperature characteristics, the character and intensity of the respiratory activities of cells and their change during differentiation, regional differences in metabolic activity, changes in the physical state of protoplasm as shown by the methods of microdissection and by the measurement of viscosity—all such processes

must be taken into account in any full consideration of ontogeny. While at present the ways are not altogether clear, it is probable that the value of tissue culture in embryology will lie in the investigation of phenomena of this kind, rather than in the working out of a topographic map of these changes, which is involved in the concept of determination.

Of other great fields in which tissue culture has found application, particularly in relation to medicine, time does not permit me to speak at any length. In pathology its most important contribution has been to the study of tumors. Early in the history of the subject it was shown that tumor cells grow well in culture media, exhibiting distinct differences from normal tissue cells. At the same time each neoplastic type was seen to grow and behave in characteristic manner. Certain tumor cells, at least, after long cultivation outside the body lose none of their virulence, for, injected back into a living animal, they readily produce new tumors of the original character. Now evidence is accumulating to show that normal body cells subjected to certain treatment while growing in vitro, may be capable of producing malignant metastasizing tumors after reimplantations into the body of a healthy individual. These facts, which could scarcely have been revealed by other methods, give witness to the unique value of the method of explantation and lend encouragement to the expectation of further important discoveries.

Résumé and conclusion.

Summarizing briefly what has been said and including certain topics not considered here at length, we may point to the following substantial contributions to biological and medical science made through the application of tissue culture and closely related methods of research:

1. A direct ocular demonstration of the embryological basis of the neurone concept.

2. A crucial proof of the myogenic theory of the heart beat.

3. A demonstration of the potential immortality and indefinite power of reproduction of somatic cells.

4. The discovery that dissociated somatic cells of animals having at least the degree of organization found in sponges and coelenterates can reconstitute themselves into normal viable individuals by a process resembling ontogeny.

5. A means for the exact study of growth in pure lines of cells, conditions influencing the process and the chemical nature of the medium required.

6. The discovery of numerous facts with regard to cell transformations that go a long way toward the solution of the vexed question of the genetic relations of blood and connective tissue elements in favor of the monogenetic or unitarian theory.

7. Important evidence regarding cell movement, the behavior of mitochondria, the mode of connection between tissue elements and their differentiation and dedifferentiation.

8. The opening of the process of cell division to direct observation, through which the peculiar movements of the cytoplasm, the duration of nuclear phases, the periodicity of the process and the influence of various external conditions upon it have been studied.

9. The discovery that tumor cells have the power of indefinite multiplication outside the body without losing their properties and that normal somatic cells may through proper treatment be rendered capable of producing malignant tumors when reintroduced into the body of an animal.

10. The direct observation of the behavior of tissue cells toward microorganisms and the conditions under which certain immune reactions occur.

11. The opening of tissue cells to the direct action of drugs whereby the influence of the latter on the living protoplasm may be studied.

Let me, however, not place too much emphasis on what has been accomplished. We are here not merely to review the past and take stock of the present but to look toward the future. From what has been said it must be clear that the actual accomplishment is but little in comparison with what remains to be done. In all of the subjects just enumerated, and in many others, further intensive investigation is needed. The time has gone by, however, when the amateur may describe a few casual experiments and still expect a hearing, for the novelty of the results and the manual dexterity involved can no longer be a cause for wonderment. The method, if it is to continue fruitful, must be applied with perseverence, thoroughness and discretion, with all necessary controls and with data sufficiently numerous to bear statistical scrutiny. The ideas underlying the method are sound, but in its application vision of the great problems awaiting solution and their precise formulation for experimental test must hold sway over mere technical skill.

A species of Proboscidiella from Kalotermes (Cryptotermes) dudleyi Banks, a termite of Central America, with remarks on the oxymonad flagellates.

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With Plates 21–24.

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I. Introduction and Historical Account.

An interesting group of Protozoa living in termites is that comprising the oxymonads, which in many ways are very different from other known flagellates. By prolongations of the anterior

ends of the bodies they may attach themselves to the lining of the intestine, but they are also able to swim about in the lumen of the canal. Having no cytostomes they ingest wood, as do many termite flagellates, through the general surface of the body.

What was probably the first member of this group to be recorded is Microrhopalodina enflata from Kalotermes flavicollis of Europe, which was described but not figured by Grassi and Foa (1911). In the usual form this organism is multinucleate, and is attached to the intestinal wall by a 'peduncle' or 'neck'. When the host moults, however, the parasite is said to become free, lose the 'neck', and develop flagella. A fuller discussion of this genus will be given later (p. 378).

Janicki (1915) described Oxymonas granulosa from Neotermes connexus ('Kalotermus castaneus') of Hawaii, giving an account of mitotic division. He noticed that this organism resembled Microrhopalodina in several respects, but separated it generically chiefly because, in contrast to Grassi's flagellate, it was usually uninucleate, though rarely binucleate.

In his monograph on the flagellates of termites Grassi (1917) figures flask-shaped flagellates from N. erytraeus and Glyptotermes parvulus which are undoubtedly oxymonads. He did not recognize that fact, however, but supposed them to be developmental stages of multinucleate flagellates of the family Calonymphidae.

Recently Kofoid and Swezy (1926 *a*, *b*) gave an account of three new species of Oxymonas, re-examined Janicki's species, correcting his account for flagella and other structural details, and described a new multinucleate oxymonad, Proboscidiella multinucleata. Later in the same year a small species of Oxymonas was recorded from Cryptotermes hermsi (Kirby, 1926).

As a result of the study of the Protozoa of termites collected in 1925 in the Canal Zone and Costa Rica, as well as of several species obtained from other localities, it is possible for the

writer to make some additions to the knowledge of this group of flagellates. These studies, as well as others still unpublished, have been facilitated by material placed at my disposal by Dr. A. E. Emerson, Dr. S. F. Light, Mr. G. F. Hill, Mr. J. E. Zetek, and Dr. L. R. Cleveland. The Central American collections were made with the aid of a grant given to Dr. L. R. Cleveland by the Bache Fund of the National Academy of Sciences, and were identified by Dr. T. E. Snyder. Many of the illustrations were prepared by Miss Lisbeth Krause.

II. Material and Technique.

The observations on living uninucleate oxymonads which are here recorded were made on material from four species of Kalotermes s. str., two of which came from Central America and two from California. Of C. dudleyi the material at my disposal was obtained from two sources: a colony (T–234) collected by Mr. Zetek in Ancon, and one (T–239) collected from furniture in Balboa, Canal Zone. Smears from the former were prepared at the Barro Colorado Island Biological Station soon after their capture; the latter were brought to New Haven and fed for variable periods on filter paper before preparations were made.

In addition to the usual methods of preparing material, fixation in osmic vapour and use of the dark-field condenser were of much assistance. The former procedure, with subsequent staining in iron haematoxylin, facilitated observations on the flagella and blepharoplasts. Unfortunately this method was not used for Proboscidiella, but fixation in Flemming's fluid without acetic acid gave comparable results. Staining in Delafield's haematoxylin after fixation in Schaudinn's fluid was very satisfactory for nuclear structure and assisted in the formation of conclusions concerning the existence of a parabasal body, for in other material on the same slides (Devescovina, Stephanonympha, and others) the parabasal was well stained.[1] The dark-field condenser fitted to a mon-objective

[1] The parabasal bodies of many flagellates in termites, including Trichomonas, Devescovina, Calonympha, and Stephano-

binocular microscope proved very helpful in determination of the number and length of flagella in the uninucleate oxymonads, but this equipment was obtained too late for use with Proboscidiella. On serial sections of the intestine of C. dudleyi the flagellates could be studied in their normal relation to the intestinal wall.

III. The Uninucleate Oxymonads.

The oxymonads have a limited distribution among termites, as they have been found only in members of one sub-family, the Kalotermitinae. In this group, however, they seem to be widespread, for an oxymonad occurs in thirty-one of the thirty-six species listed on pp. 382–3, and is not known to be absent from four of the other five. Although none has been found by the writer in Porotermes grandis, the significance of this probable exception is less because the genus Porotermes differs both structurally and in flagellate fauna from other members of the sub-family to a greater extent than these differ among themselves. In twenty-two species of these termites which the author has studied, and in six other species recorded in the literature, an oxymonad occurs which is predominantly uninucleate. In C. dudleyi this is replaced by a multinucleate form similar to P. multinucleata, and in Calcaritermes brevicollis there is, in addition to the uninucleate form, an undescribed multinucleate flagellate with an organ of attachment similar to that of Proboscidiella, but with a different form and arrangement of nuclei and body-fibres.

Observations on flagellates similar to Oxymonas from K. tabogae and K. marginipennis of Central America, and K. hubbardi and K. minor of California, were made by reflected light from the dark-field condenser. In every instance four long flagella were seen (figs. 1–9, Pl. 21). In a crushed speci-

nympha, are clearly demonstrated by staining in Delafield's haematoxylin, after fixation in Schaudinn's fluid with or without acetic acid. In the case of Metadevescovina debilis from Kalotermes hubbardi the addition of 10 per cent. acetic acid to the fixing fluid had no apparent effect on the parabasal body.

men (fig. 7) the flagella could be traced to the two blepharoplasts, from which they originated in pairs. Although individuals varied very much in length, the flagella were in all cases longer and in some more than twice as long as the body. As seen in living material, illuminated by light through the dark-field condenser, they give no impression of weakness, but on the contrary are stout and vigorous, slowly propelling organisms with anterior processes as well as others without those structures. The same number and arrangement of flagella has been determined in material from K. hubbardi and K. marginipennis fixed in osmic vapour.

In three species of Oxymonas from South American termites, and presumably also in O. granulosa, which was re-examined, Kofoid and Swezy (1926) found six short flagella, three attached to each blepharoplast. They give this number as characteristic of the genus, stating that 'this genus is fundamentally of the triflagellate type'. In accordance with this and other observations, the genus Oxymonas Janicki, 1915, is emended by them to include the two blepharoplasts, each connected by a rhizoplast to a centrosome on the nuclear membrane, and connected together by a semicircular filament, and the three flagella arising from each blepharoplast.

In features other than the flagella, the uninucleate oxymonads which the writer has observed resemble those described by Kofoid and Swezy. Because of the difference in that respect, however, it may be necessary, in agreement with the procedure followed in the case of trichomonad and other flagellates, to establish another genus for the forms with four flagella. The writer is not prepared to do this without making observations on the type species of the genus Oxymonas. It is, however, desirable to consider the quadriflagellate forms for the sake of comparing them with the new species of Proboscidiella and defining the characters of the family to which the oxymonads belong.

In addition to the value of dark-field illumination for observations on the flagella, this method facilitates study of the microorganisms adhering to the bodies of the oxymonads as well as

other flagellates of termites. The possible error, which has often been made, of confusing those organisms with flagella is thus eliminated, for their appearance and movements, when observed in this manner, are seen to be very different from those of flagella. Most of them are spiral forms, probably spirochaetes (Cleveland, 1928). By feeding the termites for a period of about ten days on filter paper soaked in 5 per cent. acid fuchsin, as recommended by Cleveland (1928), the spirochaetes may be caused to disappear from the bodies of the flagellates, as well as from the intestine of the termite. After this method had been used upon the oxymonad from K. hubbardi, rod-like forms, which had not been noticed on untreated flagellates, were found to be present.

The abundance of micro-organisms on the bodies of different individuals is, of course, very variable. The numbers vary on some species from a condition in which they cover both the body proper and the organ of attachment, to one in which there are very few or none at all. But it is possible, nevertheless, in some cases, to use the presence and abundance of certain types of micro-organisms as a specific characteristic. Thus on the oxymonad from K. hubbardi there are usually some, but not many, spirochaetes, while on that from K. panamae every individual has a dense coat of those organisms. O. pediculosa, according to Kofoid and Swezy, has a similarly large number. This point is admirably illustrated by Metadevescovina debilis from K. hubbardi, though that is not an oxymonad. On every individual of this species there are, normally, large numbers of spirochaetes, which are shorter and stouter than those on Oxymonas, forming a dense covering which has been mistaken for a coat of cilia. By feeding the host on acid fuchsin all these may be removed. On various species of uninucleate oxymonads, spirochaetes of one type, a long, narrow, much spiralled form, have been seen. As will be discussed later, this is replaced on the new species of Proboscidiella by a short, rod-like micro-organism, though longer forms are apparently present on the type species of that genus.

The above remarks on uninucleate oxymonads are included

here chiefly for comparative purposes. A detailed cytological study of the structure and mitotic division, for which material from several species is available, may be expected to clarify our knowledge of the morphology and life-history of these interesting flagellates.

IV. Proboscidiella kofoidi, sp. nov.

A multinucleate oxymonad was described by Kofoid and Swezy (1926*b*) from Planocryptotermes nocens Light, a termite of the Philippine Islands, under the name of P. multinucleata. In C. dudleyi Banks, a termite which occurs in the Canal Zone and which, like Pl. nocens, lives in dry wood and damages furniture and woodwork, the writer found a multinucleate flagellate very similar to it, yet sufficiently distinct to be assigned to a new species. In recognition of the work of Dr. C. A. Kofoid on this genus, it may appropriately be named P. kofoidi. The two flagellates correspond closely in most characteristics except the number and size of flagella and the arrangement of blepharoplasts in each unit. While in the type species there are described three very short flagella associated with each nucleus, each mastigont [1] of P. kofoidi possesses four long flagella.

1. Morphology and Behaviour.

A characteristic structure of Proboscidiella, as well as of Oxymonas, is the organelle of attachment called by Kofoid and Swezy a proboscis, but perhaps more appropriately termed a rostellum,[2] which, because of its great variability in length, is

[1] The term karyomastigont was used by Janicki for the complex in Stephanonympha and Calonympha consisting of one nucleus with the surrounding portion of the cytoplasm, the blepharoplast, the parabasal apparatus, the axial filament, and the flagella emerging from that blepharoplast. It is synonymous with the single neuromotor system with its associated nucleus, as used by Kofoid, except that this does not include the portion of cytoplasm. When the nucleus is lacking (in Calonympha) the term akaryomastigont is applied to the remaining structures, while mastigont may be used without reference to presence or absence of the nucleus, thus including both of the other terms.

[2] As used in metazoan morphology the term proboscis is applicable to

believed by them to be highly contractile. In the hind-gut of the termite flagellates may be closely crowded together, attached to the wall by these organelles (fig. 10, Pl. 22). Since in C. dudleyi, as in other termites, there is a chitinous intima lining this part of the intestine, the expanded ends of the rostella are not applied directly to the epithelial cells.

The intestinal faunas of many termites of the host species brought to New Haven and examined while living were found to consist of several species of flagellates, including Stephanonympha, Devescovina, a five-flagellated trichomonad and smaller forms, in addition to forms of Proboscidiella both with and without rostella. In shape and size the latter forms of Proboscidiella, although very variable, resembled Stephanonympha (36–96 × 31–78 microns). The flagella, which exceeded in length those of Stephanonympha, were all directed backward over the body and were in active motion, slowly propelling the organism forward. The area anterior to the nuclei, where, in other individuals, an organelle of attachment may be developed, was evenly rounded and clear. In the cytoplasm of these motile individuals were numerous spherical inclusions and particles of wood, the latter sometimes so large that the bodies were distorted to accommodate them. Rostellate forms were also abundant, though less so in this material than the motile forms. Although actively moving flagella were present on some of the rostellate individuals, others lacked those structures. The latter observation agrees to some extent with that made by Grassi on Microrhopalodina, that the attached individuals lacked flagella.

The body is usually broadly ovoidal or pyriform, narrowing

an organ, usually tubular, developed in connexion with the head if not the mouth. The numerous structures grouped under this name are of diverse natures, many functioning in relation with feeding, while others have different uses, but not that of attachment. It is somewhat misleading to use this term for a structure of a unicellular organism which is not tubular nor associated in any way with feeding, but functions for attachment. These implications are avoided by use of the term rostellum, which, besides its use in botany, is applied to median, imperforate, more or less retractile organs of attachment on the scolices of cestodes.

towards the anterior end, from which the rostellum may be prolonged (figs. 14, 16 *a*, 18, Pl. 22). In a hundred individuals preserved on slides the length, exclusive of the rostellum, ranged from 23 to 165, averaging 66 microns, and the width at the broadest part of the body from 12 to 100, averaging 46 microns. Thus the length of P. kofoidi is similar to that of those individuals of P. multinucleata measured by Kofoid and Swezy, which ranged from 25 to 160 and averaged (in sixteen cases) 72 microns. On slides made from another colony (T–234) the average size was smaller, 50 ×38 microns.

On flagellates attached to the intestinal wall by the ends of the rostella, those organelles are generally relatively short and broad, though some are longer than in the individuals selected for illustration (fig. 10, Pl. 22). The end is broadly expanded and often is concave. On smears, however, the rostella are commonly longer, ranging up to 299 microns, which in this case was nearly four times the length of the body, 77 microns. Great lengths are infrequent, the usual size being from about 10 to 120, with an average in a hundred cases of 66 microns. While often the ends of the rostella are enlarged (fig. 16 *a*, 18, Pl. 22), frequently they are pointed or truncate (fig. 11, Pl. 22). The organelles may become broken off or detached from the flagellates, as evidenced by the fact that many isolated rostella were found on smears. It is possible, then, that some of those forms lacking rostella have not retracted the process, but it has been detached. In the rostellum there are a great many fibres, prolonged from the fibrillar system of the body (fig. 24, Pl. 23).

While oxymonads described or observed in twenty-eight species of Kalotermitinae are uninucleate, with occasional binucleate and rarely multinucleate stages, the two members of the genus Proboscidiella are predominantly multinucleate. In P. multinucleata uninucleate stages have been described, but the writer has found none in P. kofoidi. The smallest number of nuclei in several thousand individuals of the latter species was two, the largest number twenty-six (fig. 33, Pl. 24). In the latter instance the nuclei had separated into two groups of seven and nineteen, and binary fission had been proceeding.

Numbers above eleven are, however, infrequent. Though every count from two to nineteen, seldom more, occurs, eight is the most common number. This is borne out by records presented in the accompanying table from three hundred individuals on twelve slides. Of these sixty-five, which is by far the highest single number, had eight nuclei; the median number is eight and the average number in the whole group, disregarding the two exceptional cases of twenty-two and twenty-six, is 8·04.

FREQUENCY IN NUMBER OF NUCLEI IN 300 INDIVIDUALS.

2 – 4	12 – 7
3 – 7	13 – 6
4 – 18	14 – 5
5 – 24	15 – 1
6 – 42	16 – 7
7 – 39	17 – 3
8 – 65	18 – 2
9 – 27	19 – 2
10 – 20	22 – 1
11 – 19	26 – 1

On smears fixed in the Canal Zone soon after the termites of colony T–234 were secured, the bodies of Proboscidiella were almost without exception crowded to the base of the rostellum with yellow-brown spherules, some of which occurred among the blepharoplasts and fibrils anterior to the nuclei (fig. 16 *b*, Pl. 22). These spherules measured from 1 to 5, most commonly between 3 and 4 microns in diameter, and each was enclosed in a clear area, apparently a vacuole. While most of them were quite spherical in form, many were somewhat irregular in outline (fig. 17, Pl. 22). Sometimes these latter were irregularly ovoidal, in some there was a flattening or even a depression at one side, and in a few cases the globules were as irregular in outline as starch grains. The interior was not uniform in appearance, but seemed to be filled with a few large or many small vacuoles, and sometimes to contain also a few granules. When the body is ruptured these globules may remain intact after release.

In termites of another colony (T–239) which were brought to New Haven and fed for several days on filter paper, the cytoplasmic inclusions of Proboscidiella were not the same

(fig. 11, Pl. 22; fig. 25, Pl. 23). There were fewer and smaller spherules, which did not have the same yellow-brown colour. They were usually not stained, but in material fixed in Flemming's fluid there were among them some, similar in outline to the others, which stained deeply with iron haematoxylin, as well as some with a deeply staining shell and inner granules (fig. 20, Pl. 23). Without doubt the filter-paper feeding had altered the cytoplasmic inclusions, so that the normal situation is not presented by flagellates from hosts which had been thus treated.

Spherules similar to those of this species of Proboscidiella are abundant in O. granulosa and occasionally, at least, occur in P. multinucleata, and in some species of Calonympha and Stephanonympha. In O. pediculosa Kofoid and Swezy found in many individuals large, spherical granules which stained deeply. Janicki suggests that in O. granulosa the spherules may be derived directly from the particles of wood, which acquire a rounded form. The facts which are known all point to the conclusion that they are derived from the ingested wood, perhaps as an extracted substance. Their lack of uniformity in internal structure makes it unlikely that they are fluid reserve products.

Among the spherules, in flagellates from normally fed termites, are small wood particles, the cytoplasmic fibres, often large fragments of wood, and tubular structures of variable thickness and length within which spherules may be enclosed. In a specimen the hosts of which had been fed upon filter paper for six days, there was, at the end of the bundle of axostyles (fig. 15, Pl. 22) a curious tubular structure of different nature. The tube was stained with iron haematoxylin, and thus is comparable with similar but shorter tubes in some other specimens (fig. 11, Pl. 22). The writer has been unable to form an opinion as to the nature of these structures. They are similar to certain cytoplasmic inclusions in P. multinucleata, which Kofoid has suggested may be 'vestigial sleeves' handed down from an Oxymonas-like ancestor.

A narrow zone of ectoplasm is distinct in some (fig. 11, Pl. 22) but not in all specimens. This seems to be alveolar in nature, as

many fine lines, which are probably alveolar walls, traverse it. Inwardly the ectoplasmic layer, in which no cytoplasmic inclusions occur, is distinct from the endoplasm, and outwardly it is bounded by a thin pellicle.

Flagellates have been found in which there is a broad layer of granular protoplasm surrounding the body and, in some cases, partly covering the rostellum. When this substance covers an individual which lacks a rostellum, there is some resemblance to a cyst. The structure of this zone, which varies in width, is, however, not that of a cyst wall, but rather that of disintegrating protoplasm. A similar layer separating the pellicle from the wood-containing cytoplasm occurs in some individuals of Devescovina, Stephanonympha, and Calonympha, which are probably degenerate. In the intestine of the host, some moribund flagellates are most likely normally present, and the manipulations of smear-making certainly produce degenerative changes in some specimens. Consequently, although several specimens similar to that drawn by Kofoid and Swezy as a cyst of P. multinucleata have been found, these do not seem to the writer to afford convincing evidence for the occurrence of encystment in P. kofoidi.

A striking difference between the two species of Proboscidiella concerns the micro-organisms adherent to the surface of the body. In P. multinucleata the authors state (p. 311) that ' the surface of the body is generally covered with a dense coat of vertically attached bacteria ', and their figure shows rather long, rod-like forms. Possibly these are spiral organisms similar to those of certain species of Oxymonas, as these often appear like rods in fixed material. On the body of P. kofoidi there are instead short, slightly curved, rod-like micro-organisms. In a few cases these are absent, in others there are a few limited chiefly to the posterior region, while in many cases they are abundant on both the body and rostellum (fig. 19, Pl. 22). When sufficiently destained, the interior of these rods is clear, except for several large chromatic granules. The rods vary in length, and some are in stages of transverse fission. The micro-organism, which is also present in the gut contents,

has not been observed in the living condition, but it is similar to a form, abundant in K. hubbardi, which moves with rapidity. The surface of Joenia annectens is generally covered with similar organisms in about the same numbers as those on P. kofoidi.

The nucleus is spherical or more frequently broadly ovoidal with the longer axis parallel to the longitudinal axis of the cell. Within the delicate membrane the nuclear material is arranged in a manner similar to that of Oxymonas (figs. 12, 13, Pl. 22). Most of the space is occupied by granules of chromatin somewhat unequal in size and arranged in groups or strands. There seldom, if ever, is a regular distribution of isolated granules on a reticulum. In the posterior region of the nucleus is a clear space in which is the karyosome surrounded, frequently, by a number of radial strands. The karyosome is somewhat irregular in outline; often it is broadly ovoidal with the longer axis transverse to the longer axis of the nucleus. The periphery is often more deeply stained, like a shell, and in the interior may be clear spaces. Sometimes there seems to be a central granule (figs. 34 *a*, 35, Pl. 24), such as that shown in P. multinucleata, but it is by no means certain that the structure really exists. At any rate there is so far no convincing evidence that a centriole exists within the karyosome. Besides the karyosome there may sometimes, but not often, be one or more similar masses in the nucleus.

The nucleus measures from 4 to 9, usually 6 to 7, microns in diameter, thus being of about the same size as that of the type species. The karyosome, however, is proportionately larger than in that flagellate. While, according to the text (Kofoid and Swezy, 1926, p. 304) the diameter of the karyosome in their species is not greater than one-fifth of the diameter of the nucleus, and is often smaller, that of this form usually measures fully a third and ranges from a quarter to a half of the nuclear diameter. On a set of smears prepared from T–234 the nuclei were smaller, 4 to 5 microns, and the karyosome diameter 0·2 to 0·25 of this. While less than that in the other race, this is still greater than that of P. multinucleata.

The fibrillar, or neuromotor, system consists of the same elements described by Kofoid and Swezy, but the arrangement of some of these is different. The numerous fine cytoplasmic fibres (figs. 11, 18, Pl. 22), termed by Kofoid retractor fibres, are not limited to the peripheral cytoplasm, as in P. multinucleata, but extend longitudinally throughout the endoplasm. Apparently they begin in the rostellum, where they constitute some of the rostellar filaments, run past the nucleus, and approach close to the posterior end of the body. Sometimes several of these cytoplasmic fibres are collected into a bundle.

Peripheral to the cytoplasmic fibres are the axostyles (fig. 20, &c., Pl. 23), which usually equal the nuclei in number, though often there are several more. Excepting in small individuals, in which the rostellum is short or absent, the axostyles do not reach the posterior end of the body, and may stop far short of this. Each axostyle is flattened, lies just under or in the ectoplasm, and is often turned outward at the posterior end to approach the pellicle, through which the tip may project. There is often some variation in the proportional length and thickness of axostyles in the same individual, and there may be considerable variation in this respect between those of different individuals (cf. figs. 11, 18, Pl. 22). After bending around the upper side of the nucleus, the axostyle tapers abruptly close beneath one of the blepharoplasts and gives origin to several fibres which continue into the rostellum (figs. 28, 29, Pl. 23). The axostyle seems to be composed of fibres, for some are split into several or divided completely up to the region of the blepharoplasts (figs. 22, 23, Pl. 23). In some cases the posterior end has a structure like that of the axostyle of Oxymonas, an arrow-shaped enlargement with a ring around the broader portion, and the part adjacent to this may be divided into several fibres (fig. 14, Pl. 22). It seems probable that the posterior portion, just described, has been lost in most instances, leaving only the tapering, more deeply staining portion (cf. p. 374).

It is by no means certain that the axostyle-like structures in Proboscidiella and Oxymonas are of the same nature as the axostyles of Trichomonas and Devescovina. They

rather resemble the 'axostyles' of Pyrsonympha and Dinenympha, and perhaps the 'chromatic basal rod' of Devescovina. Like these, they stain deeply with iron alum haematoxylin, and not with Delafield's stain; like that of Pyrosonympha they may break up into fibres.

In the arrangement of blepharoplasts and flagella P. kofoidi differs from the type species. In the latter, according to the authors, 'the centrosome lies directly on the anterior face of the nuclear membrane. From it there passes anteriorly a short, delicate rhizoplast to the blepharoplast bar, from whose proximal end, constituting the primary blepharoplast, arises the single primary flagellum, while from the distal end, the secondary blepharoplast, emerges the pair of equal, secondary flagella'. The length of the flagella is shown as not more than three times the diameter of the nucleus, which is about six microns.

In P. kofoidi there are, in each mastigont, arising in pairs from the two blepharoplasts, four flagella of a length (about 60 microns) often equal to or exceeding that of the body (fig. 11, Pl. 22; figs. 20, 25, 27, Pl. 23). In this number and relative length of flagella this oxymonad agrees with several species of uninucleate oxymonads which the writer has observed (p. 359). Thus each single mastigont of the multinucleate form agrees in this particular with the uninucleate form, as is to be expected from the probable close evolutionary relationship between these flagellates.

In each mastigont the two blepharoplasts, which are some distance apart, are unequal in size and shape (fig. 27, Pl. 23). The larger primary blepharoplast, which is close to the anterior end of the nucleus and lies over the axostyle at the point where this narrows abruptly (figs. 28, 29, Pl. 23), is elongated and more pointed at the end opposite to that from which the two flagella arise. From this pointed anterior end there extends into the rostellum a fibre, the proximal section of which, when stout and deeply stained as it frequently is, appears as an oblique bar (fig. 27, Pl. 23). Situated more anteriorly and to the left of the primary blepharoplast is the more spherical secondary blepharoplast, from which also a fibre extends toward the rostellum. In

most cases this fibre seems to meet that from the other granule, so that the two blepharoplasts seem to be at the ends of a fork unequal in length and thickness (figs. 27–9, Pl. 23), but since the fibres often run parallel as far as they can be traced, it is probable that they are not actually united. From the posterior end of each blepharoplast, at the outer edges as seen from above, two flagella emerge (figs. 27–9, Pl. 23). From the smaller secondary blepharoplast a filament runs posteriorly; sometimes this seems to meet the membrane of the adjacent nucleus (figs. 30–2, Pl. 23); sometimes it apparently ends freely (fig. 26, Pl. 23).

The fine interconnexions between axostyle, blepharoplasts, and nucleus are much more difficult to determine than are those structures described in the preceding paragraph, so that, although it is possible for the writer to describe them from a few observations, the description may not be entirely accurate. Under the primary blepharoplast the axostyle seems to end in a small enlargement which is connected to the blepharoplast by a very short strand (fig. 26, Pl. 23). Between this blepharoplast and the nucleus there is possibly a rhizoplast (fig. 26, Pl. 23), which seems to end in a granule at the point where it meets the membrane. In contrast to the stout blepharoplast bar described in P. multinucleata is the delicate strand which may sometimes be made out connecting together the two blepharoplasts (fig. 26, Pl. 23).

A brief summary of the above account will indicate how the blepharoplasts, axostyle, and nucleus of each mastigont are connected. The primary blepharoplast is perhaps attached by a rhizoplast to a granule (centrosome ?) on the nuclear membrane, to the axostyle by a short strand, and to the secondary blepharoplast by a fine filament. This blepharoplast also gives rise to a stouter filament which passes into the rostellum. From the secondary blepharoplast a similar filament also enters the rostellum, and another passes posteriorly to meet the membrane of the nucleus of the mastigont to the left, or to end freely between this and its own nucleus. To the membrane of that nucleus it may adhere, at some distance from the anterior end, in a manner similar to that in which the rhizoplast bands of

Staurojoenina are attached. Thus, by this and the primary rhizoplast, may the nuclei be suspended in position, if this account is correct.

No parabasal bodies similar to those of many other polymastigote flagellates have been found in P. kofoidi, despite careful search in material treated by methods which clearly demonstrated those structures in Devescovina and Stephanonympha on the same slides. In the position which the parabasal body would, if present, occupy a group of granules has sometimes been seen (figs. 25, 31, Pl. 23; fig. 11, Pl. 22). These granules are often in one or two rows, or grouped in a triangular form close to the nucleus near the primary blepharoplast. Similar granules may exist anterior to the nuclei, or scattered about in the anterior part of the body (fig. 25, Pl. 23), but the frequent observation of a group of these in the position mentioned suggests that they constitute a definite structure. Although not quite the same as the parabasal bodies of many other flagellates, this structure is probably of similar nature. No true parabasal body has been seen, to the writer's knowledge, in any oxymonad flagellate.[1]

2. Fission and Mitosis.

Multiplication by fission of the body, preceded by separation of the nuclei into two groups, unaccompanied by mitosis, occurs in P. kofoidi as well as in P. multinucleata. The numbers of nuclei in the two groups are generally unequal. Only one instance of equal separation was observed, with six nuclei in each set. Of those with unequal groups, at one extreme was a case in which there were two and three, and at the other extreme one with nineteen and seven nuclei (fig. 33, Pl. 24). In the earlier stages the nuclei have formed two groups, the axostyles are deeper in the body than usual, and the proximal, but not the distal, end of the rostellum is split, or at least the rostellar fibres are separated. Later the rostellum splits to the

[1] Since writing this the author has observed in a species of Oxymonas from Kalotermes clevelandi, fixed in Flemming's fluid without acetic acid, a granular structure exactly comparable to that of P. kofoidi.

tip, and finally the two processes and two groups of nuclei are at opposite ends of the body, preliminary to final separation. In one case three groups of nuclei were seen in one body, an instance, probably, of multiple fission.

Although a good many instances of mitotic organization have been found, these were limited to a few stages. The earliest changes begin while the nuclei are in their usual position. The chromatin draws away from the membrane, the karyosome moves toward the centre, and the strands of chromatin granules become thicker and more closely packed (fig. 35, Pl. 24). These changes occur synchronously in all nuclei of an individual, and are most frequent on slides where there are other stages of mitotic organization. There are instances of degenerating nuclei in which the chromatin is similarly clumped, but these occur in only one or two nuclei of a group and are different in appearance from the prophase organization stages.

In later stages the rostellum is shorter and the nuclei have migrated from their usual position into the posterior cytoplasm (fig. 34, Pl. 24). Blepharoplasts, axostyles, cytoplasmic fibres and flagella remain in place. It has not been possible to determine whether or not a centrosome, lying upon the membrane, migrates with the nucleus.

In the migrating prophase nuclei (fig. 34, Pl. 24) the chromatin is dispersed and organized into groups and threads of chromatin granules, perhaps following a contraction stage such as that previously described. The karyosomes, which have moved toward the centre of the nuclei, are of the usual size and form, some showing an inner clear region in which, especially under low magnification, there sometimes seems to be a granule. More careful observation, however, renders the existence of this granule doubtful.

Mitosis proceeds in the nuclei free in the cytoplasm, as in Lophomonas, Calonympha, Stephanonympha, and Oxymonas (fig. 37, Pl. 24). Apparently the number of dividing nuclei at least frequently approximates eight. Thus, in six cases of karyokinesis, in which the nuclei had migrated from their usual position, there were 8, 8, 7, 7, 7, and 6 of these. In a

number of others the bodies had been ruptured, the old fibrillar apparatus torn away, leaving in the remaining cytoplasm 10, 7, 7, and 4 nuclei.

The mitotic figures are very different from those of any other flagellates of termites which have been described, except Oxymonas. In the earliest spindle stage observed (fig. 36, Pl. 24), the nuclear membrane, which is more distinct than in the resting stage, is only slightly elongated (about 8×7 microns). Within the membrane the most conspicuous structure is the curved centrodesmose, which is about 1·5 microns broad. Where the truncate ends of this touch the membrane, that is indented as if drawn inward. Surrounding the centrodesmose is chromatic material in the form of numerous short, varicose strands or linear groups of granules.

Later the nucleus becomes more elongated and its ends more pointed. No longer is the membrane drawn inward at the ends of the centrodesmose, but rather it is pushed outward. The centrodesmose, which, when sufficiently destained, seems to consist of a stainable shell with a clear core, has become more bowed (figs. 39, 42, Pl. 24), as if its increase in length has been greater than that of the nucleus. The convex margin may appear stouter and stain more deeply, as if there were a coarse, stainable strand there (fig. 42, Pl. 24), but that is not always the case. In some cases there is an indication of a central strand (fig. 40, Pl. 24), but this may be an illusion due to uneven staining of the shell.

Surrounding the centrodesmose is the spindle, with fibres converging to the poles. While the bulk of the spindle is on the concave side of the centrodesmose, it also extends around the convex side (fig. 39, Pl. 24). Apparently there are no definite chromosomes; at least, if there are, these have no regularity of form and are very numerous. In the mass of chromatic material there are some single granules, but more frequently these granules are collected into linear groups (figs. 37*a*–42, Pl. 24). The chromatin belt may extend continuously from pole to pole, may be more restricted to the equatorial region, or may be separated into two belts as in an anaphase.

In many instances, and possibly in all, there is a mass on the spindle comparable in size to the karyosome (figs. 41, 42, Pl. 24). This mass is often irregular in form, or may be spherical and much like the karyosome of the resting nucleus in appearance, even to the apparent inner granule. In some later spindles this body seems to be constricting in a dumb-bell form.

At about this time there has begun to differentiate what is most likely the new axostyle (figs. 40, 43, Pl. 24). This is a deeply staining strand, tapering to a point at each end and in length about half that of the nucleus. One pointed end is close to the end of the centrodesmose, from which it is separated by the nuclear membrane and a small space in the cytoplasm. No granule nor rhizoplast has been detected at the end of this structure, even in material prepared in such a manner as to demonstrate clearly the blepharoplasts.

A flagellate without a rostellum (fig. 14, Pl. 22), with nuclei and axostyles different in structure from those of most other specimens, may be interpreted as in a stage following mitotic division. The axostyles differ from the usual condition in each having a posterior prolongation consisting of several filaments which finally collect into an arrow-shaped enlargement. Besides the karyosome, many of the nuclei contain one or two other masses similar in size, form, and stainability. It is possible that the posterior prolongation of the axostyle is later lost, and the nucleus takes on the more typical resting appearance. In the cytoplasm of this flagellate are several structures which seem to be degenerating nuclei, as if all the products of mitosis had not survived.

While this series of stages is too incomplete for the solution of several problems of fundamental importance, it makes possible an incomplete interpretation of the type of mitosis. In P. kofoidi nuclear division proceeds in the same general manner as in Oxymonas, in which, however, there are generally more definite chromatin strands. Although no direct evidence as to the origin of the centrodesmose has been obtained, it is probable that this develops from the karyosome, as in Euglenoidina and, according to Janicki, in O. granulosa.

The origin of the karyosome-like body seen on the spindle is obscure. At the time of mitosis the blepharoplasts, flagella, axostyles, and fibres are discarded, and probably new organelles are differentiated.

The existence of an extranuclear division centre in the oxymonad flagellates, which is to be expected, awaits conclusive demonstration. None has been recorded in Oxymonas nor in Proboscidiella, excepting that Kofoid and Swezy found a granule which they refer to as a centrosome on the nuclear membrane of P. multinucleata and two such granules on the nucleus of O. projector. They were unable, however, to follow the fate of these granules during nuclear division. It has not been possible to detect any centrosomes nor paradesmose in mitotic figures of P. kofoidi, but the material has been limited. Mr. C. C. Zeliff has obtained evidence of the existence of centrosomes and a delicate paradesmose in a uninucleate oxymonad from Rugitermes kirbyi.

3. Racial Differences.

Most of the observations recorded in this paper were made on P. kofoidi from termites of colony T–239, but upon comparison with those from T–234 several differences, most of which have already been mentioned, were detected. On slides prepared in the Canal Zone from the latter colony, the flagellates were smaller, the organelles of attachment longer, the average number of nuclei was less, the nuclei were smaller, the spherical cytoplasmic inclusions were different in number and size, and the body-surface was free of the micro-organisms so characteristic of the others (fig. 16, Pl. 22). While in the flagellates from T–239 the average size was 66 × 46 microns, and forms exceeding 100 microns in length of the body proper were not uncommon, in those from the other colony the average was only 50 × 38 microns, and no individuals of exceptionally large size were observed. Long rostella were more common than in the others, and the average length of twenty of these was 50 per cent. greater. In one case the rostellum was nearly five times the length of the body (220 and 45 microns). In a hundred indivi-

duals the number of nuclei ranged from 2 to 11, averaging 5·97 and with 6 as the median number. Besides being less numerous, the nuclei were smaller in size, 4 × 5 microns, and the karyosomes measured only 0·2 to 0·25 of this. The cytoplasm of these flagellates was, as already mentioned, crowded with large spherules (p. 364), while in the Proboscidiella examined from termites of colony T–239 these were smaller and less numerous, although under normal conditions they may have been the same. The presence of micro-organisms on flagellates from the termites brought to New Haven was a constant characteristic, but these were absent from the other material.

It is, of course, possible that transportation and filter-paper feeding produced changes in the intestinal flagellates, but it seems unlikely that those factors would modify any of the characteristics referred to except the spherules in the cytoplasm. In comparison with what may occur in other flagellates of termites treated in a similar manner, it is probable that the unusual conditions would, if anything, decrease the size and stimulate division, besides altering the cytoplasmic contents.

These differences in the flagellates from the two host colonies may represent racial peculiarities which have developed during a long period of isolation, or may be within the normal range of variation. At any rate, this demonstration of the differences which may occur shows the need of caution in the classification of similar flagellates, for, if the hosts had been of two species, specific separation of the two forms of Proboscidiella would have seemed justifiable. As it is, it seems best to regard the smaller flagellate as a variety of the species kofoidi, of which the type is the form from colony T–239.

4. Systematic Position of Species.

The differences between the flagellar apparatus of those species of uninucleate oxymonads mentioned in the first part of this paper, and those species of Oxymonas described by Kofoid and Swezy (1926), lead to some confusion as to the characters of the group. The former agree in number and

relative length of flagella with each mastigont in P. kofoidi, while the latter have twice the number possessed by each neuromotor unit of P. multinucleata. A constant difference in the number of flagella is justification for the separation of genera in many flagellates. No doubt this should also be the case with the oxymonads, unless further study shows that there is greater uniformity than present descriptions seem to indicate. Although the writer was inclined at first to assign the Central American species of multinucleate oxymonad to a new genus, further consideration of the close resemblance in most respects between it and the described species of Proboscidiella made it seem more likely that it should rather be placed in that genus. If that is the correct procedure, it is at least necessary to modify the characters of the genus as regards number of flagella to permit the inclusion of this species with four flagella in each mastigont.

5. Diagnosis: Proboscidiella kofoidi sp. nov.

Entozoic flagellates with the characters of the genus, but differing from the generic type in having no blepharoplast bar but two blepharoplasts possibly connected by a delicate filament, and two pair of long flagella in each mastigont. The number of mastigonts varies from two to nineteen or more, averaging eight. The body normally contains numerous characteristic spherules, as well as particles of wood. The flagellates are generally attached to the lining of the gut by an anterior prolongation of the body, but may lack that structure and swim in the lumen by means of the long flagella. The average sizé is about 66 × 46 microns, and the average length of the rostellum about that of the body. Short, rod-shaped micro-organisms are often attached in large numbers to the surface of the body, by one end.

Host: Kalotermes (Cryptotermes) dudleyi Banks, 1918. Colony T–239. Locality: Balboa, Canal Zone, Panama.

Variety in Kalotermes (Cryptotermes) dudleyi. Colony T–234. Locality: Ancon, Canal Zone, Panama.

(For characteristics see p. 375.)

V. The Genus Microrhopalodina and Grassi's Flask-shaped Forms of Calonymphidae.

The account of Microrhopalodina enflata given by Grassi and Foa (1911) is very incomplete and lacks illustrations, but it is probable that they were describing a multinucleate oxymonad. This flagellate, according to them, has a brief period of free existence, and a much longer period of attachment to the intestinal lining by a long 'peduncle'. In the fixed stage the flagella are lost, growth occurs, a flask-form is assumed, and the nuclei, blepharoplasts, and axostyles multiply repeatedly, forming a group at the base of the ' neck '. From near the nuclei the axial filaments may continue anteriorly to the attached extremity of the neck, and many may run posteriorly into the body. In this stage solid wood is ingested and there are in the cytoplasm numerous round corpuscles which, though not fat, are possibly some reserve material. When the termite moults, the flagellates become detached, the neck disappears, the characteristic corpuscles and wood are discarded, and flagella develop. This form may then divide into small, uninucleate flagellates, which give origin to new attached flask-forms.

From this description, since we receive no information concerning size, number, or structure of nuclei, number or length of flagella, arrangement of blepharoplasts or size of the body, it is impossible to gain a clear conception of Microrhopalodina. Of those flagellates in termites which are known, Proboscidiella agrees most closely with the description, in that the nuclei are located in both at the base of the 'neck ', for the most part, rostellar and body-filaments are present, the anterior organ of attachment may be well developed or lost, flagella may develop, especially on free forms, and characteristic corpuscles as well as wood particles may be present in the body. Perhaps a re-study of Microrhopalodina will show that it has the same structure as Proboscidiella, in which case the latter name will become a synonym of the former, which will be the type genus of the family.[1]

[1] Through the kindness of Dr. A. E. Emerson, of the University of Pittsburg, the writer has been able to examine two living nymphs of

The uninucleate, rostellate flagellates figured by Grassi in 1917 were regarded by him as stages in the life-history of Calonymphidae, to which family he also assigns Microrhopalodina. The so-called flask-forms of S. silvestrii var. neotermitis erythraei resemble Oxymonas, while the larger flask-forms attributed to Diplonympha foae, which have more highly developed rostella but only one or sometimes two nuclei, are not unlike O. pediculosa and the form from K. panamae. If any more than morphological evidence is needed to show the error, which Bernstein (1928) has pointed out, in Grassi's interpretation of these flagellates, this is supplied by the fact that in K. minor and K. hubbardi, which harbour uninucleate oxymonads, no Calonymphidae nor other multinucleate flagellates are present.[1]

The family Calonymphidae, in which Grassi placed the above-mentioned Protozoa, is defined by him as comprising flagellates with many nuclei, many axial filaments, blepharoplasts, and parabasal bodies. While Proboscidiella would be covered by that definition, except for the lack of definite parabasal bodies, Oxymonas would not, and obviously Oxymonas cannot be separated from Proboscidiella. Because of this fact, together with the marked morphological differences, the oxymonad flagellates should be removed from the family Calonymphidae, in which they have recently been included by Bernstein (1928).

VI. The Family Oxymonadidae.

The oxymonad flagellates, which form a unified group with characteristic structures and peculiar distribution, are set apart in several respects from all other flagellates known at present.

K. flavicollis. These were infected with an abundance of Joenia annectens, Hexamastix termitis, Janickiella grassii, and the so-called Trimitus-forms, but no specimens of Microrhopalodina were found.

[1] Grassè (1926, p. 572) states that he has found the uninucleate stages of M. enflata in K. flavicollis, and accepts Grassi's interpretation of similar flagellates as developmental stages of Stephanonympha.

It is therefore very desirable that a family be established to contain them, in order that they may no longer be confused with quite different forms. For convenience in classification the writer proposes the new family Oxymonadidae.

1. Diagnosis: Oxymonadidae fam. nov.

Entozoic flagellates which have an anterior organelle of attachment, the rostellum, possess a characteristic nucleus, and have a peculiar type of mitosis. The nucleus contains a large karyosome and granular chromatin filling the remaining space. During division, there is a stout centrodesmose, which is apparently developed from the karyosome. There are in each mastigont two blepharoplasts, each of which gives rise to a group of flagella (two or three in all cases except P. multinucleata), an axostyle, probably a parabasal (see p. 371), and cytoplasmic and rostellar filaments. The majority of known species are uninucleate, but there are also multinucleate forms of some of these species and multinucleate species. So far as is known at present these flagellates occur only in termites of the sub-family Kalotermitinae, in which they are widely distributed.

2. List of Genera and Species.

(?) Microrhopalodina Grassi and Foa, 1911.

M. enflata Grassi and Foa, 1911.

Host: Kalotermes (Kalotermes) flavicollis Fabr.
Locality: Italy.

(Inadequately described and not figured.)

Oxymonas (Janicki, 1915) emend. Kofoid and Swezy, 1926.

O. granulosa Janicki, 1915.

Host: Kalotermes (Neotermes) connexus Snyder, 1922.
Locality: Honolulu, Hawaii.

O. projector Kofoid and Swezy, 1926.

Host: Kalotermes (Glyptotermes) perparvus Emerson, 1925.
Locality: Kartabo, British Guiana.

O. pediculosa Kofoid and Swezy, 1926.

Host: Kalotermes (Lobitermes) nigriceps Emerson, 1925.
Locality: Kartabo, British Guiana.

O. gracilis Kofoid and Swezy, 1926.

Host: Kalotermes (Rugitermes) magninotus Emerson, 1925.
Locality: Kartabo, British Guiana.

O. parvula Kirby, 1926.

Host: Kalotermes (Cryptotermes) hermsi Kirby, 1925.
Locality: Fanning Island, Central Pacific Ocean.

Proboscidiella Kofoid and Swezy, 1926.

P. multinucleata Kofoid and Swezy, 1926.

Host: Kalotermes (Planocryptotermes) nocens Light, 1921.
Locality: Manila, Philippine Islands.

P. kofoidi Kirby, 1929.

Host: Kalotermes (Cryptotermes) dudleyi Banks, 1918.
Locality: Balboa, Canal Zone, Panama.

3. Systematic Position of Family.

Wenyon (1926) separates the monozoic, diplozoic, and polyzoic flagellates as Protomonadida, Diplomonadida, and Polymonadida. In this scheme the Oxymonadidae have no place, for the family includes both monozoic and polyzoic forms, and there would certainly be no justification for distributing Oxymonas and Proboscidiella into separate orders. For them it is better to retain the order Polymastigida, as does Calkins (1926), which includes the polyzoic Calonymphidae, besides other flagellates. Therefore, although in nuclear structure and mitotic division the Oxymonadidae differ from other Polymastigada, they may be assigned to that order, near the Dinenymphidae.

VII. Appendix: List of Kalotermitinae Examined for Protozoa.

Preparations from the following termites of the sub-family Kalotermitinae are available to the writer for the study of intestinal Protozoa.[1]

Kalotermes (Calcaritermes) brevicollis Banks, 1918. Barro Colorado Island, Canal Zone.

Kalotermes (Calc.) emarginicollis Snyder, 1926. Estrella, Costa Rica.

*Kalotermes (Cryptotermes) brevis Walker, 1853. Porto Rico.

Kalotermes (Crypt.) breviarticulatus Snyder, 1926. Taboga Island, Panama.

Kalotermes (Crypt.) dudleyi Banks, 1918. Balboa, Canal Zone.

Kalotermes (Crypt.) hermsi Kirby, 1925. Fanning Island.

Kalotermes (Glypt.) barbouri Snyder, 1924. Barro Colorado Island, Canal Zone.

Kalotermes (Kalotermes) clevelandi Snyder, 1926. Ancon, Canal Zone.

Kalotermes (K.) contracticornis Snyder, 1925. Cartago, Costa Rica.

*Kalotermes (K.) flavicollis Fabricius. Europe.

*Kalotermes (K.) hubbardi Banks, 1920. California.

Kalotermes (K.) immigrans Snyder, 1922. Fanning Island.

*Kalotermes (K.) jouteli Banks, 1920.

Kalotermes (K.) marginipennis Latreille, 1811. Cartago, Costa Rica.

Kalotermes (K.) minor Hagen, 1858. California.

Kalotermes (K.) panamae Snyder, 1924. Barro Colorado Island, Canal Zone.

*Kalotermes (K.) schwarzi Banks, 1920.

Kalotermes (K.) tabogae Snyder, 1924. Taboga Island, Panama.

Kalotermes (Lobitermes) longicollis Banks, 1918. Taboga Island, Panama.

Kalotermes (Neotermes) holmgreni Banks, 1918. Taboga Island, Panama.

Kalotermes (Rugitermes) kirbyi Snyder, 1926. Cartago, Costa Rica.

[1] For the material from those species which are starred, I am indebted to Dr. A. E. Emerson, Dr. S. F. Light, Mr. G. F. Hill, and Dr. L. R. Cleveland. In preparation of this list I have been assisted by Dr. T. E. Snyder.

*Porotermes (Porotermes) grandis Holmgren, 1912. Victoria, Australia.
*Kalotermes (Cryptotermes) species. Gardner Island, Galapagos.
*Kalotermes (K.) species. Gardner Island, Galapagos.
Kalotermes species. Estrella, Costa Rica.

Oxymonad flagellates have been found in all of these excepting Porotermes grandis. In addition, oxymonad flagellates have been found in the following species, as recorded in the literature :

Kalotermes (Glypt.) perparvus Emerson, 1925. Kartabo, British Guiana.
Kalotermes (Glypt.) parvulus Sjöstedt. Africa, near Gold Coast.
Kalotermes (Neotermes) erytraeus Silvestri. Eritrea.
Kalotermes (Neo.) connexus Snyder, 1922. Honolulu, Hawaii.
Kalotermes (Lobitermes) nigriceps Emerson, 1925. Kartabo, British Guiana.
Kalotermes (Planocryptotermes) nocens Light, 1921. Manila, Philippine Islands.
Kalotermes (Rugitermes) magninotus Emerson, 1925. Kartabo, British Guiana.

The following species have been investigated for Protozoa but Oxymonadidae have not been recorded. They are not, however, known to be absent.

'Epicalotermes aethiopicus Silvestri.' Eritrea.
Kalotermes (Crypt.) havilandi Sjöstedt. Africa, near Nigeria.
Kalotermes (Glypt.) iridipennis Froggatt. Australia.
Porotermes (Porotermes) adamsoni Froggatt. Australia.

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DESCRIPTION OF PLATES 21–24.

All figures were drawn with a camera lucida at magnifications of 1,440 and 2,740, excepting figs. 10, 16 *a*, 18, and 25. The methods of fixation and staining used for each specimen are indicated in the description of the figure as follows: S., Schaudinn's fluid; F., Flemming's fluid without acetic acid; D., Delafield's haematoxylin; H., Heidenhain's iron haematoxylin.

Plate 21.

Figs. 1–9.—Uninucleate oxymonad flagellates with four flagella.

Drawings were made with the camera lucida from dark-field preparations of living material. All figures, except fig. 7, were drawn to the scale of microns. ×900.

Figs. 1–2.—From K. hubbardi. Oxymonads lacking rostella, with four flagella and attached spirochaetes.

Fig. 3.—From K. hubbardi. Spirochaetes removed by feeding host with acid fuchsin; some rod-shaped bacteria adherent to body.

Fig. 4.—From K. hubbardi. A small rostellate individual, with four flagella and adherent spirochaetes.

Fig. 5.—From K. minor. Large number of attached spirochaetes. Flagella not shown.

Fig. 6.—From K. minor. Outline drawing showing flagella.

Fig. 7.—From K. minor. Anterior end of a crushed but still living individual, showing two flagella from each blepharoplast, axostyle, and nucleus. ×1,712.

Fig. 8.—From K. marginipennis.

Fig. 9.—From K. tabogae.

Plate 22.

Figs. 10–19.—*Proboscidiella kofoidi* sp. nov.

Magnification as stated. Figs. 10 and 19 drawn to adjacent scales; fig. 16 *a* to scale of fig. 19; figs. 11, 12, and 17 to scale at top; others to scale at lower left.

Fig. 10.—Section of intestine of *Cryptotermes dudleyi*. Flagellates attached by rostella to wall. ×490. S.D.

Fig. 11.—Full length of flagella; body filaments; granules in position of parabasal body and at base of rostellum. ×900. S.H.

Fig. 12.—Resting nucleus *a* is a median optical section; *b* a surface view. ×1,712. S.H.

Fig. 13.—Resting nucleus. ×1,712. S.H.

Fig. 14.—Individual without rostellum. Arrow-shaped enlargements at ends of axostyles, before which the axostyles are split into fibres. Several possibly degenerating nuclei in cytoplasm. ×900. F.H.

Fig. 15.—Rostellum absent, axostyles and filaments gathered into bundle. Tubular structure at end of bundle not connected to axostyles. ×900. S.H.

Fig. 16.—Variety from colony T–234. *a*, Very long rostellum, expanded at tip. ×375. S.D. *b*, Same organism, showing large spherical corpuscles crowding cytoplasm. ×900. S.D.

Fig. 17.—Spherical corpuscles similar to those of fig. 16 *b*. Note clear areas within and depressions on one side of some. ×1,712. S.D.

Fig. 18.—Large individual, long rostellum expanded at tip, only basal portion of flagella shown. Spherical corpuscles, fibrillar system, and filter-paper fibres in body. ×375. S.H.

Fig. 19.—Micro-organisms adherent to surface of body. These are present generally, but have been omitted from most figures. ×900. S.H.

Plate 23.

Figs. 20–32.—*Proboscidiella kofoidi* sp. nov.

Figs. 20–3 and 25 were drawn to scale on left. ×900. All others to scale of microns on right. ×1,712.

Fig. 20.—Full length of flagella; characteristic corpuscles in cytoplasm, and some which stain deeply. S.H.

Fig. 21.—Three nuclei; characteristic form of rostellum. The flagella are omitted. S.H.

Figs. 22–3.—Splitting of the axostyles into fibres. Fig. 22 is focussed on the lower portion, fig. 23 on the upper of the same specimen. Blepharoplasts and rostellar filaments also shown. F.H.

Fig. 24.—End of a broad rostellum, showing rostellar filaments. S.H.

Fig. 25.—Diagrammatic typical figure, showing rostellum, flagella, axostyles, rostellar filaments, cytoplasmic filaments, corpuscles and in-

gested particles in body, and deeply stained granules in position of parabasal body and elsewhere.

Figs. 26–32.—Detailed diagrams showing arrangement of nuclei, blepharoplasts and associated filaments, and origin of flagella.

Fig. 26.—Rhizoplast, interblepharoplast filament, connexion between axostyle and blepharoplast, and filament from secondary blepharoplast to nucleus. F.H.

Fig. 27.—Group of nuclei and blepharoplasts, showing flagella and filaments from blepharoplast to rostellum, one of which is stout at base. Axostyles omitted. S.D.

Figs. 28–9.—Origin of flagella, axostyles with anterior prolongations, filaments from blepharoplasts into rostellum. F.H.

Fig. 30.—Filament from secondary blepharoplasts to nuclei. F.H.

Fig. 31.—Same, and collection of granules in position of parabasal body. F.H.

Fig. 32.—Filament from secondary blepharoplast to adjacent nucleus. F.H.

PLATE 24.

Figs. 33–43.—*Proboscidiella kofoidi* sp. nov. Fission and mitosis.

The drawings of entire organisms (figs. 33, 34, 37) were drawn to scale near top. ×800. Those of individual nuclei by scale in lower left. ×1,522.

Fig. 33.—Fission. The nuclei have separated into two groups of 19 and 7, and the rostellum has divided. S.H.

Fig. 34.—Nuclei in early prophase, migrating into posterior region of cytoplasm. S.H.

Fig. 34 *a*.—Nucleus from fig. 34.

Fig. 35.—Condensed chromatin mass. Probably earlier than fig. 34. Flagellates may be seen in which all the nuclei, in the usual position near the blepharoplasts, are in this condition. S.H.

Fig. 36.—Later prophase nuclei in portion of ruptured body. Note indentations in membrane at ends of centrodesmose. S.H.

Fig. 37.—Later prophase nuclei, migrated from anterior region. The blepharoplasts are in their usual position. S.H.

Fig. 37 *a*.—Single nucleus from fig. 37.

Fig. 38.—Hollow appearance of centrodesmose; spindle fibres and chromatin belt. S.H.

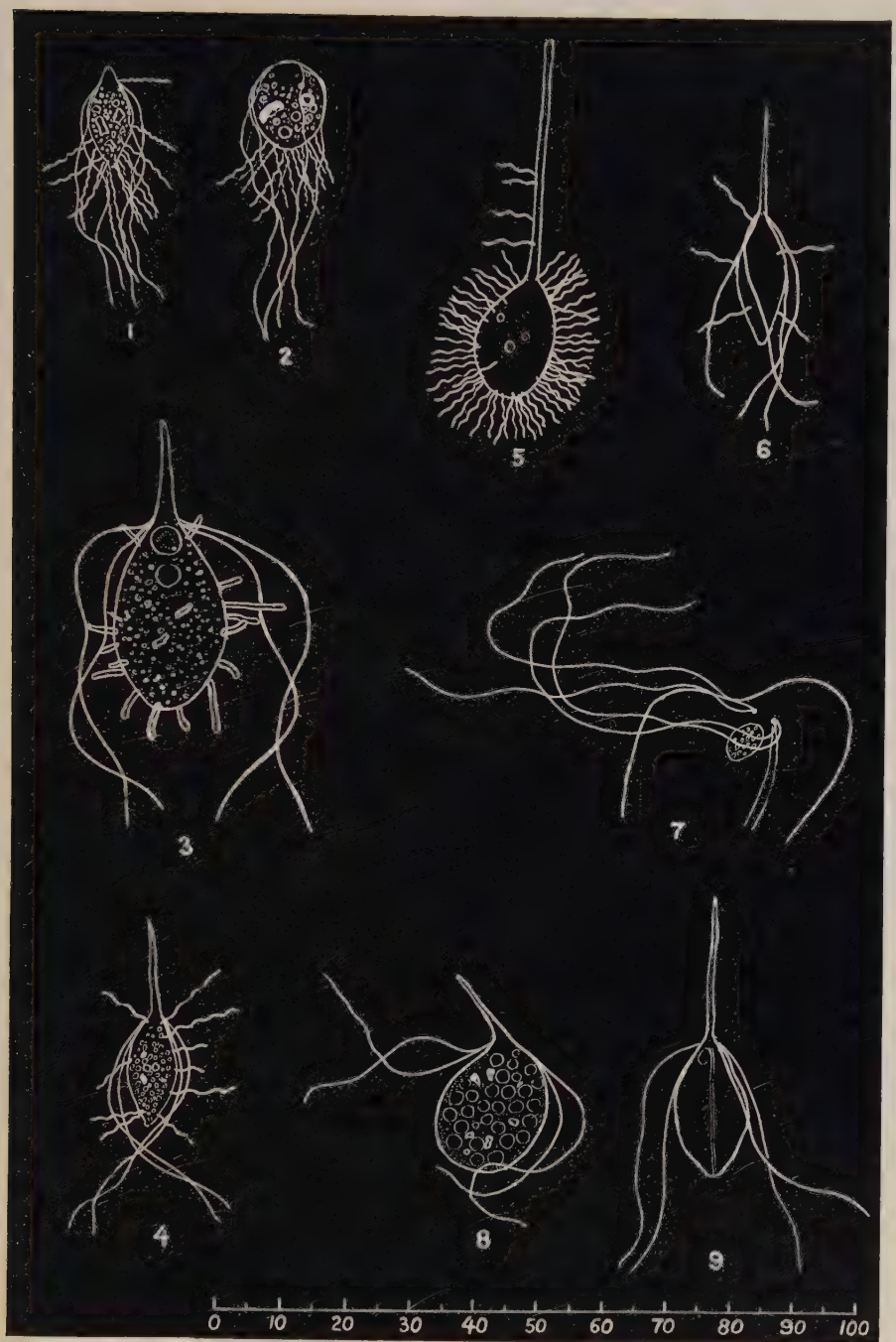
Fig. 39.—Increased curvature of centrodesmose. S.H.

Fig. 40.—Appearance of new axostyle (?) at one pole. S.H.

Fig. 41.—Chromatic mass on spindle. Aggregations of chromatin granules separating into two groups. S.H.

Fig. 42.—Similar. Hollow appearance of centrodesmose; denser stain on convex side; chromatic mass on spindle; groups of chromatin granules collecting toward poles. S.H.

Fig. 43.—Spindle-formed nuclei; development of new axostyles. S.H.



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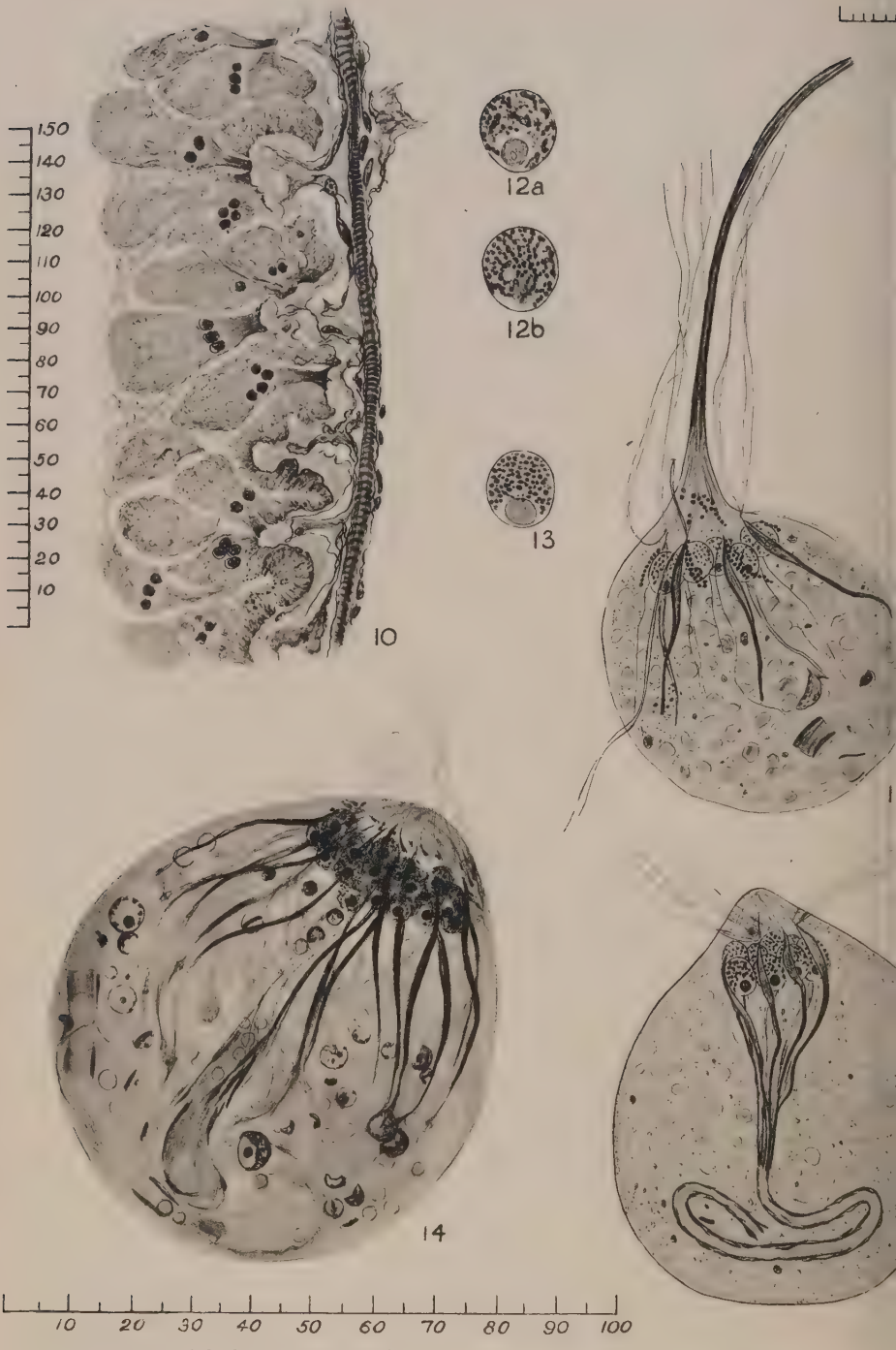
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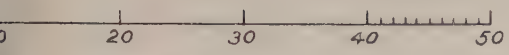

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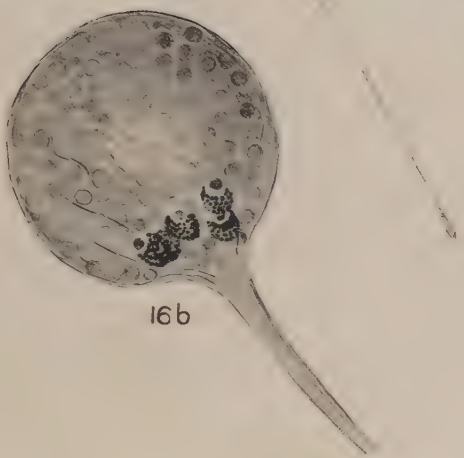

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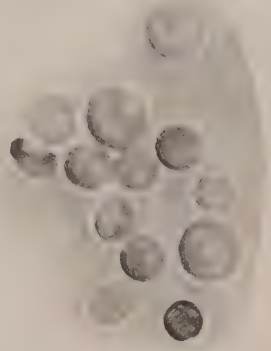

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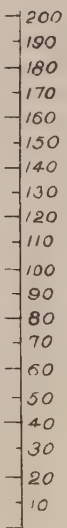

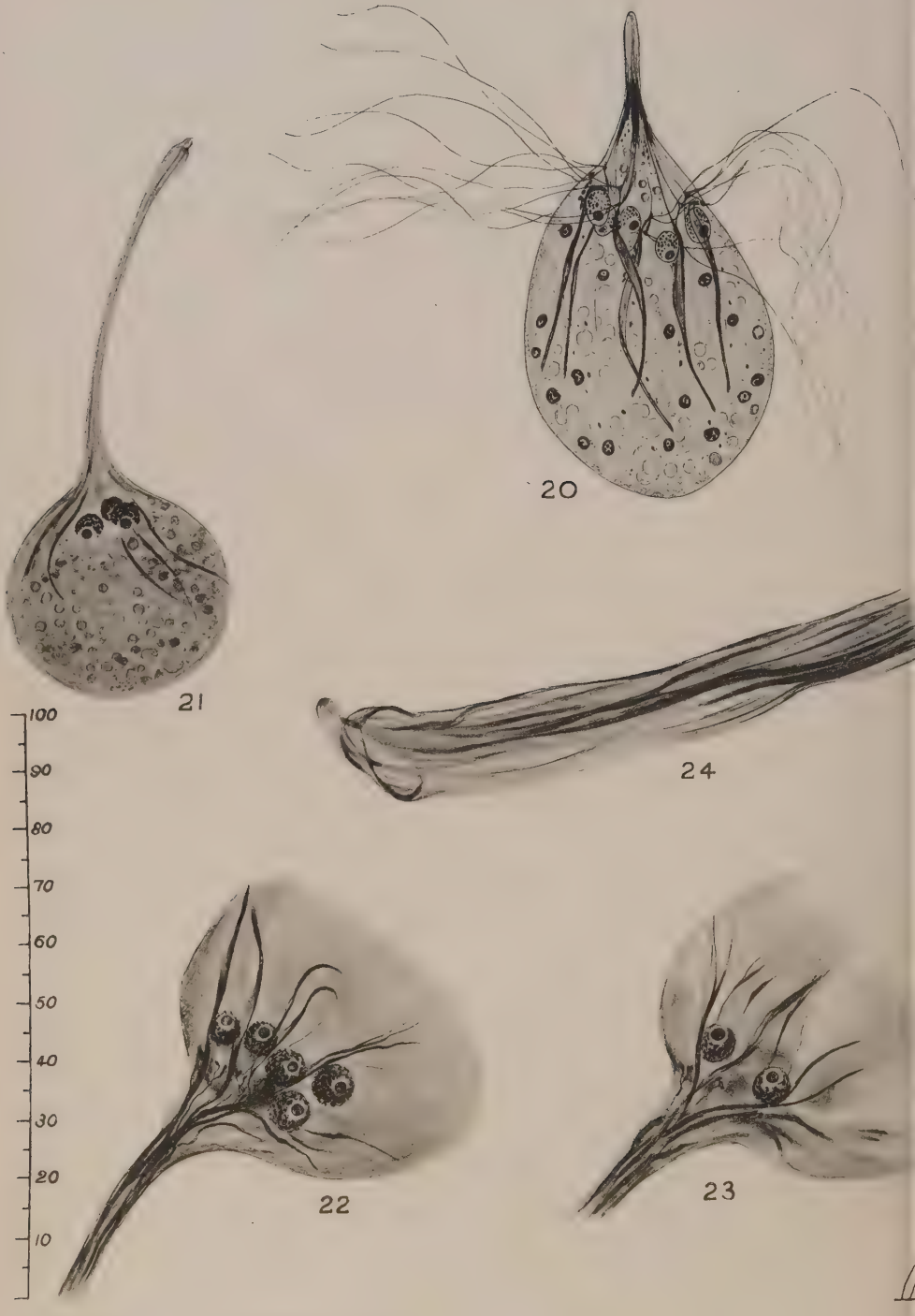

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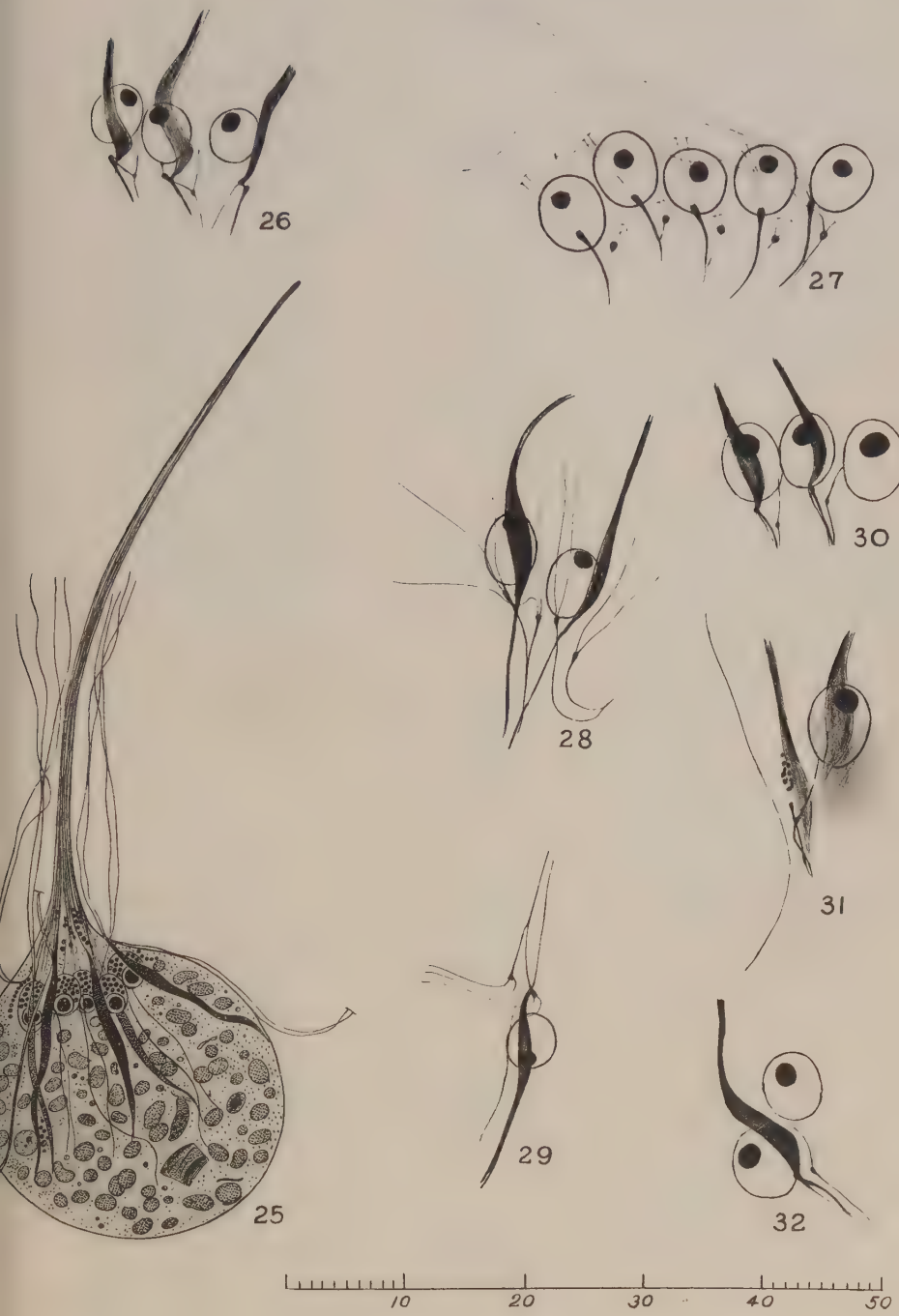
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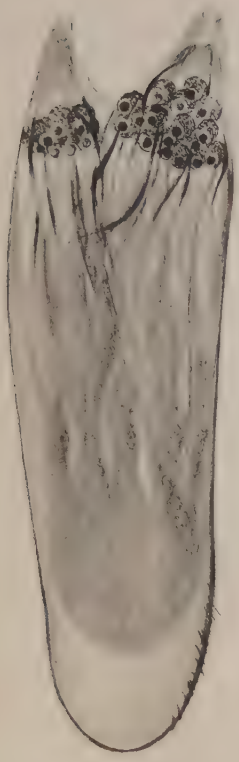
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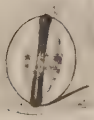
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